# GENERATION OF IMPROVED *E.coli* STRAINS TO BE USED IN THE CONSTRUCTION OF LIGAND LIBRARIES

ZEHRA SEDA ELMACI

May, 2005

# GENERATION OF IMPROVED *E.coli* STRAINS TO BE USED IN THE CONSTRUCTION OF LIGAND LIBRARIES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

**MASTER OF SCIENCE**

in Biotechnology

by
Zehra Seda ELMACI

May 2005
İZMİR

We approve the thesis of **Zehra Seda ELMACI**

**Date of Signature**

5 May 2005

.......................................................
**Assist. Prof. Dr. Alper ARSLANOĞLU**
Supervisor
Department of Biology
Izmir Institute of Technology


5 May 2005

.......................................................
**Assoc. Prof. Dr. A. Fazıl YENİDÜNYA**
Co-supervisor
Department of Molecular Biology and Genetics
Cumhuriyet University


5 May 2005

.......................................................
**Assist. Prof. Dr. Canan TARİ**
Co-supervisor
Department of Food Engineering
Izmir Institute of Technology


5 May 2005

.......................................................
**Assist. Prof. Çağlar KARAKAYA**
Department of Biology
Izmir Institute of Technology


5 May 2005

.......................................................
**Assist. Prof. Dr. Gülşah ŞANLI**
Department of Chemistry
Izmir Institute of Technology


5 May 2005

.......................................................
**Prof. Dr. Şebnem HARSA**
Head of the Biotechnology and
Bioengineering Program
Izmir Institute of Technology


.......................................................
**Assoc. Prof. Dr. Semahat ÖZDEMİR**
Head of the Graduate School

# ACKNOWLEDGEMENTS

# ABSTRACT

The first step in the construction of ligand libraries is the total cloning of gene fragments coding millions of ligand variants into selected plasmid vectors. Since ligand proteins are expressed in fusion with the phage pIII protein, they can be incorporated into the newly synthesized phage particles in bacteria. The other proteins that make up the phage particle are supplied by the super infection of bacteria containing the ligand DNA clones with the helper phage. The diversity of the ligand libraries is directly proportional to the number of different gene fragments. In the current phage display technology, there are some drawbacks which can dramatically influence the diversity of a given ligand library. One of the drawbacks arises from the fact that, theoretically half of the phage particles, which are produced after super-infection, can carry only the helper phage genome instead of the ligand gene, even though they display a specific ligand protein. These phages can compete with those which carry both the ligand gene and its protein during the selection process and consequently they loose the ligand protein during the second round of selection. In any given library, this problem can cause the loss of rare but functionally very important ligands during the sequential selection procedure. Another drawback is the reinfection of a super-infected bacteria during or after the super-infection. This can increase the frequency of those phage particles which only carry the helper-phage genome, in the total phage population. These two disadvantages in the phage display technology are due to super-infection. This study aimed at the elimination of the super-infection step from the phage display technology by the insertion of M13 phage genome excluding intergenic region which contains the DNA sequences necessary for replication instead of uses of helper phage. To accomplish this purpose, Homologous Recombination method was used. It is an *in vivo* method for the replacement, deletion or insertion of sequences in bacteria.

After Homologous Recombination, three colonies were observed and observed colonies were exposed some confirmation tests. First step of these tests was related to the presence of unique recombination cassette sequences in chromosomal DNA.

Results we obtained showed that the presence of such fragments in chromosomal DNA. However, the functional test of the M13 genes in *E.coli* chromosome suggested the toxicity of an unidentified M13 gene products on *E.coli* chromosome

# ÖZET

Ligand (Peptide) Kütüphanenelerinin yapımınındaki ilk adım, milyonlarca ligand varyantlarını kodlayan gen fragmanlarının, seçilmiş plasmid vektörlere total olarak klonlanmasıdır. Ligand proteinler, plasmid vektörde bulunan faj pIII filament proteiniyle füzyon olarak eksprese edildiklerinden, bakteride yeni faj partiküllerinin yapısına girerler. Faj partiküllerini oluşturan proteinler de ligand DNA klonlarını içeren bakterilerin yardımcı fajlarla (helper phage) super-enfeksiyonu ile sağlanır. Ligand kütüphanelerin zenginliği, içerdikleri farklı gen fragmanları sayısıyla doğru orantılıdır. Faj displey yönteminin şu anki durumuyla, kütüphane diversitesini negatif yönde etkileyebilecek bazı yönleri vardır. Bunlardan bir tanesi, super-enfeksiyondan sonra oluşan faj partiküllerinin teorik olarak yarısının, spesifik bir ligandı taşıdıkları halde ligand geninin bulunduğu plasmidin yerine yardımcı fajın genomunu taşıyor olmalarından kaynaklanmaktadır. Bu fajlar hem bir ligandı hem de genini taşıyan fajlarla, seçim sırasında rekabet ederler ve bir sonraki seçim sırasında taşıdıkları ligandı da kaybederler. Bu durum, herhangi bir kütüphanede en az sıklıkta temsil edilen fakat fonksiyon bakımından büyük öneme sahip olabilecek ligandların ardışık seçim aşamalarında kaybolmalarına neden olmaktadır. Diğer bir dezavantaj, süper-enfeksiyon sırasında veya sonrasında yardımcı faj tarafından enfekte olan bir bakterinin yeniden enfeksiyona uğramasıdır. Bu da, ligand taşımayan faj partiküllerinin sayıca artması nedeniyle, ligand DNA'sını taşıyan faj partiküllerinin popülasyondaki sıklığını azaltır. Faj gösterim yönteminde, bu iki dezavantaj faj süper-enfeksiyonundan kaynaklanmaktadır. Bu çalışma DNA replikasyono için gerekli olan genler arası bölgeyi içermeyen M13 genomunu yardımcı faj kullanımı yerine *E.coli* kromozomuna ekleyerek bu sürecin faj display yönteminden eliminasyonunu öngörmektedir. Bu amacı gerçekleştirmek için Homolog rekombinasyon methodu kullanıldı. Bu metod bacterinin kromozomundaki genlerin değiştirilmesi silinmesi ve eklenmesi için kullanılan *in vivo* bir methottur. Homolog recombinasyondan sonra, 3 koloni gözlenmiş ve bazı doğrulama testlerine maruz bırakılmıştır. Bu testlerin ilk adımı rekombinasyon kasetinde bulunan blirli genlerin varlığını doğrulamaya ilişkindir. Elde ettiğimiz sonuçlar bazı genlerin kromozom üzerinde bulunduğunu göstermiştir. Fakat , M13 genlerinin *E.coli* kromozomunun üzerindeki fonksiyonları belirlenmemiş M13 genlerinin toxic etkisi nedeniyle fonksiyonlarını kaybetmeye neden olan kromozomal yeniden düzenlemeyle sonuçlanmıştır.

*to Hasan ELMACI…*

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| **(+)** | **:** | Viral strand of DNA |
| **(-)** | **:** | Complementary strand of DNA |
| **bp** | **:** | Base pair |
| **C** | **:** | Complementary |
| **cDNA** | **:** | Complementary DNA |
| **C-Terminal** | **:** | Carboxy-terminal |
| **dNTP** | **:** | Deoxynucleotide triphosphate |
| **dsDNA** | **:** | Double-stranded DNA |
| **DNA** | **:** | Deoxyribonucleic Acid |
| **EDTA** | **:** | Ethylenediamine tetra acetic acid |
| **Fab** | **:** | Fragment antigen binding |
| **h** | **:** | Hour |
| **kb** | **:** | Kilobases |
| **KDa** | **:** | Kilodaltons |
| **kV** | **:** | Kilovolts |
| **m** | **:** | Minute |
| **mM** | **:** | Milimolar |
| **µl** | **:** | Microliter |
| **µg** | **:** | Microgram |
| **µF** | **:** | Microfarads |
| **N-terminal** | **:** | Amino terminal |
| **OD** | **:** | optical density |
| **pg** | **:** | pico-gram |
| **psi** | **:** | $1.05 \text{ kg/cm}^2$ |
| **PI** | **:** | Protein I |
| **PII** | **:** | Protein II |
| **PIII** | **:** | Protein III |
| **PIV** | **:** | Protein IV |
| **PV** | **:** | Protein V |
| **PVI** | **:** | Protein VI |

| | |
|---|---|
| **PVII** | **:** Protein VII |
| **PVIII** | **:** Protein VIII |
| **PIX** | **:** Protein IX |
| **PX** | **:** Protein X |
| **PCR** | **:** Polymerase Chain Reaction |
| **RF** | **:** Replicative-form |
| **s.s.DNA** | **:** Single-stranded DNA |
| **ssb** | **:** Single-stranded binding |
| **scFv** | **:** Single-chain variable fragment |
| **TAE** | **:** Tris Acetate EDTA |
| **TE** | **:** Tris EDTA |
| **Tet** | **:** Tetracycline |
| **U** | **:** Unit |
| **UV** | **:**UltraViolet |
| **(v/v)** | **:**Volume/volume |
| **(w/v)** | **:**Weight/volume |

# CHAPTER 1

# INTRODUCTION

## 1.1. Phage Display

## 1.1.1. Overview of Phage Display

Phage Display is described briefly as an extremely new and remarkable molecular technique for identifying and creating ligands (polypeptides) with novel properties and changing the properties of existing one.

This method basically depends on a gene encoding ligands that are fused to phage coat protein genes and these "fusion genes" are incorporated in bacteriophage particles that also display the heterologous proteins on their surfaces (Sidhu, 2001). Phage displaying ligands with a desired binding specificity can be selected from library pools by binding to an immobilized ligand (Sidhu, 2000). This selection can be performed *in vitro* and *in vivo* (Johns *et al*., 2000; Sparks *et al*., 1996; McCafferty and Johnson, 1996). *In vitro* selection of phage displayed ligands can be screened not only against a wide range of biological targets but also inorganic ones (Whaley *et al.,* 2000).

G.P. Smith firstly defined this powerful technology in 1985 as a tool, which provides a direct relationship between phenotype and genotype. The main advantage of this technology is illustrated in Figure 1.1 (Azzazy *et al*, 2002).

immobilized target

phenotype of
binding protein

pIII

tip of phage
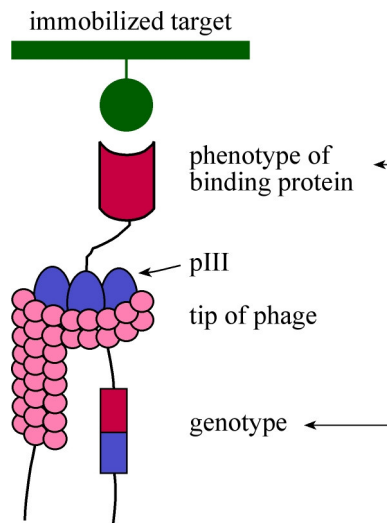
genotype

**Figure 1.1.** Schematic representation of the direct link between genotype and phenotype

Phage display technology is composed of three steps. The first step is the DNA library construction, second step is the preparation of phages displaying ligands and the last step is the selection of ligands with desired properties (Wiersma *et al*., 2003).

Phage Display library is generated by the insertion of the genes, encoding proteins to be displayed, into the phage genome and expressed together as a fusion protein. Following expression the ligand fused with the phage coat protein is displayed on the filamentous phage. Phage particles displaying ligands with desired properties are then selected by exposing the phage library to an immobilized target molecule. This process is achieved by simple, rapid and inexpensive molecular biological techniques. Phage display technology had a major impact on immunology, cell biology, drug discovery, pharmacology, and plant science. Although, this technology is being used routinely in many areas, the success of this technology initially depends on the quality of the constructed library and researches towards the improvement of the technology are still going on (Baek et al., 2002; Sidhu, 2000).

## 1.1.2. Filamentous Phages

Bacteriophages are viruses that infect bacteria, which are also called phages. Most phages consist simply of nucleic acid enclosed within a protein capsid (also named coat). The genome may be dsDNA, ssDNA, dsRNA or ssRNA (Russel et al,

2004; Sambrook et al., 1989). Phages as cloning vectors have been used for a lot of purposes up to now (Russel et al., 2004). Bacteriophages such as Lambda, T7 and filamentous phages (Rodi and Makowski, 1999; Bleaks, 2001; Smith et al., 1985; Sidhu, 2001)) are used in phage display technology. However, filamentous phages are the most preferred and used vectors in this technology due to some of their biological properties (Russel et al., 2004). Their genome is small hence easier to manipulate and the genome tolerates insertions into non-essential regions. They can be produced in high amounts and the phage particles are stable under broad range of potential selection conditions (Russel *et al.,* 2003).

## 1.1.2.1. Structure of Filamentous Phages

The filamentous phages are viruses that infect *E.coli,* they have a circular single stranded DNA genome, which is covered with a protective coat. M13, f1, fd are filamentous phages and they are all genetically and phenotypically closely related to each other. The genomes of these bacteriophages have been completely sequenced (Beck *et al.* 1973; Van Wezenbeek *et al.* 1980; Beck and Zink 1981; Sambrook *et al.* 1989). Their DNAs are 98 % homologous (Sambrook et al., 1989)

M13 phage is one of the most well known filamentous phage. They are 7-nm.in diameter. The length of the enclosed genome is 900 nm. Their protein coat is composed of PVIII, (also called major coat protein). At one end of the M13 phage particle, PIII and PVI proteins and at the other end of the particle PVII and PIX proteins are located (Figure 1.2). The entire genome of M13 phage consists of 11 genes and an intergenic (IG) region which contains the replication of origin for (+) and (-) strand synthesis (Figure 1.3). Two of the phage proteins (X-V) are required to generate s.s. DNA, other three (I-XI-IV) are necessary for phage assembly and the remaining five (III-VII-VI-IX-VIII) are components of the phage particles which are also named as coat proteins (Table 1.1) (Russel *et al.* 2004, Sidhu 2001). All five-coat proteins are responsible for host cell recognition and structural stability but PIII is also necessary for host cell recognition and infection. Although, PIII is structurally the most complex coat protein it is the most well-characterized coat protein. This protein consists of three distinct domains. N-terminal domain is responsible for the translocation of the viral DNA into *e.coli*, the second domain confers host cell recognition by binding to the F pilus present

on the bacterium and the C-terminal domain is responsible for the integration of PIII into phage coat (Sidhu, 2001).
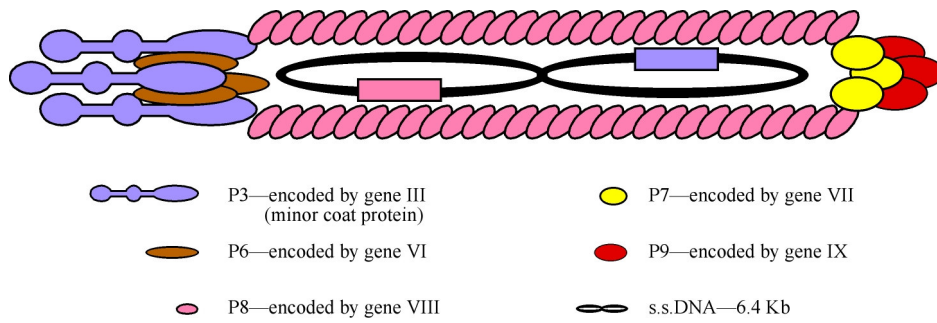


**Figure 1.2:** Structure of M13 Filamentous Phage with its coat proteins.
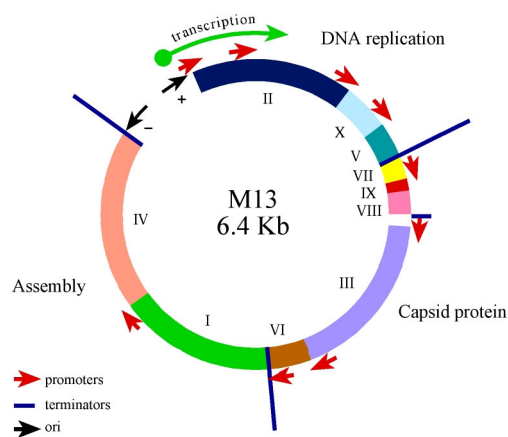


**Figure 1.3:** M13 Genome

**Table 1** F-specific filamentous phage genes/proteins and properties

| Gene | Protein | Size (aa) | Function | Location | Used for display? |
|------|---------|-----------|----------|----------|-------------------|
| I | I | 348 | Assembly | Inner membrane | |
| | XI | 108 | Assembly | Inner membrane | |
| II | II | 409 | Replication (nickase) | Cytoplasm | |
| | X | 111 | Replication | Cytoplasm | |
| III | III | 406[a] | Virion component | Virion tip (end) | Yes (N-term) |
| IV | IV | 405[a] | Assembly (exit channel) | Outer membrane | |
| V | V | 87 | Replication (ssDNA bp) | Cytoplasm | |
| VI | VI | 112 | Virion component | Virion tip (end) | Yes (C-term) |
| VII | VII | 33 | Virion component | Virion tip (start) | Yes (N-term) |
| VIII | VIII | 50 | Virion component | Virion filament | Yes (N- and C-term) |
| IX | IX | 32 | Virion component | Virion tip (start) | Yes (N-term) |

[a] Mature protein without signal sequence.

## 1.1.2.2. Life Cycle of M13 Phage

PIII protein which is encoded by gene III attaches the *E. coli* pilus to initiate the infection. While PVIII strips off, PIII remains attached until the phage genome is transferred into the host cell. In the cytoplasm, the infecting (+) strand s.s. DNA is converted into a d.s. circular form which is called as "replicative form" (RF) DNA by cellular enzymes present in the host cell (host RNA and DNA polymerase and topoisomerase) (Sambrook *et al.,* 1989, Russel *et al.,* 2003) (Figure 1.4).
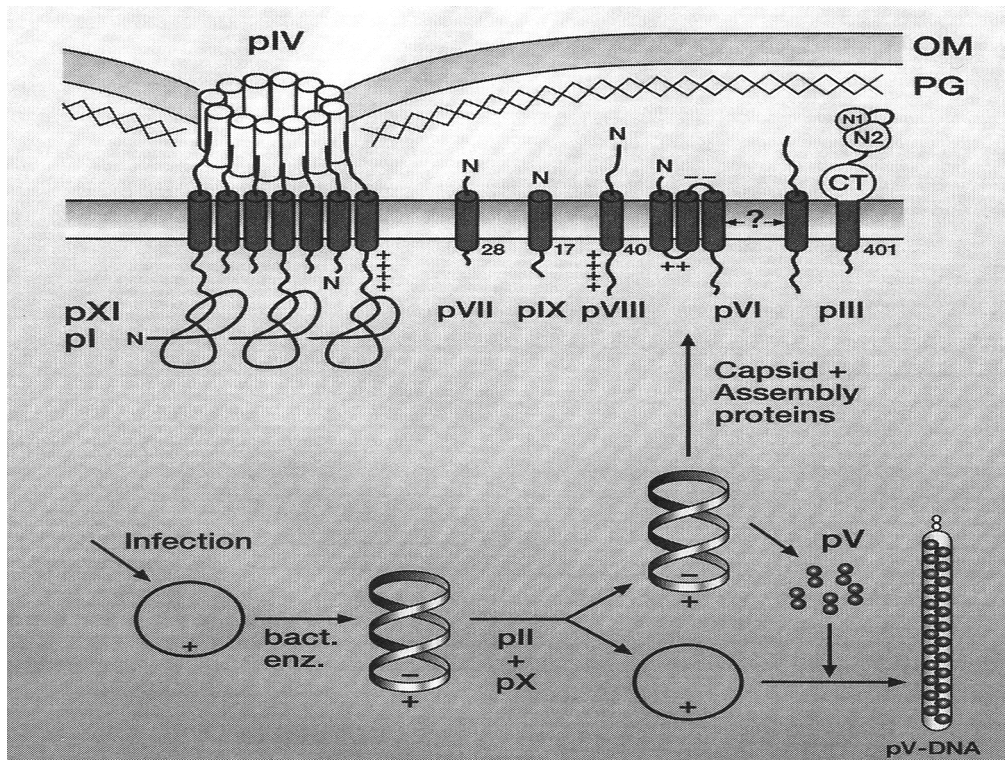
**Figure 1.4:** DNA replication, protein synthesis, and protein location in the M13 bacteriophage. DNA replication represents in the bottom of the figure. The top of the figures shows the position of the proteins in the bacterial periplasm, cytoplasmic membrane and outer membrane (OM). PG refers to the peptidoglycan layer (Source: Scott *et al.*, 1997).

Transcription of viral genes begins and proceeds in the same direction. Amplification of viral genome begins when the PII introduces a nick at a specific site in the (+) strand of the d.s. RF DNA. *E.coli* DNA polymerase III extends the (+) strands using (-) strand as template. After one round of the replication, the PII nicks again to release a linear (+) strand by *E. coli* Rep helicase. Linear (+) strand is then circularized and progeny (+) strands are converted to d.s. RF form, which serve as template for transcription by RNA polymerase (Russel *et al.* 2004).

During the accumulation of RF DNA the amount of PV, which is a ssDNA binding protein, translated from phage transcripts increases up to a critical level which then binds to the newly synthesized ss (+) sense genome to prevent the polymerase activity and block their conversion to RF DNA (Azzazy et al., 2002; Russel et al., 2004). This genome is then transferred to the cell membrane where phage assembly is initiated. Here, while PV proteins are stripped off the DNA, PVIII proteins, which are localized to cellular membrane, replace PV proteins and cover the phage DNA. The

other coat proteins which are also imbedded in the inner membrane are incorporated to the assembling phage particles and the completed phage particles are released to the outside from the cell membrane (Sidhu *et al.*, 2003).

## 1.1.3. Phage Display Technology

Display of ligands on phage surface is achieved by the fusion of genes encoding desired proteins to phage coat protein genes. Displayed proteins on phage surface are then simply chosen by affinity selection methods. (Smith and Petrenko, 1997).

In general, the process can be divided into three sections: creating a library of polypeptides, screening and selection of clones and analysis of the selected clones. (Hoogenboom *et al.*, 1998). Initially, a library of different genes encoding polypeptides is created and cloned into phage genome as fusion to coat protein gene. For this purpose, DNA encoding polypeptides is inserted between the signal sequence of the coat protein and the amino terminus of the mature coat protein by molecular biological techniques and these are then expressed. Strategies of how the genes encoding desired foreign polypeptides are fused to coat proteins are shown in Figure 1.5.
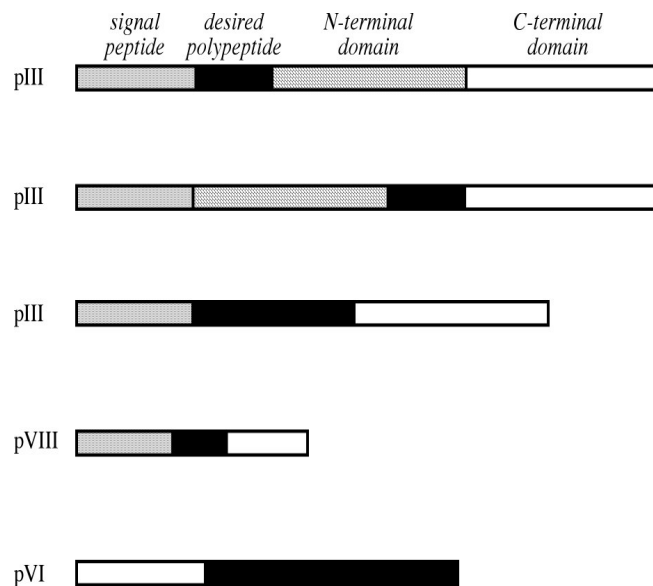


**Figure 1.5:** How the genes encoding desired foreign polypeptides are fused to coat proteins

During the screening of libraries cloned polypeptides carried on phage surface fused with coat proteins are exposed to immobilized target molecules. Phages with appropriate specificity are then captured. This process is called as affinity selection (bio-panning) (Hoogenboom, 1997; Hoogenboom *et al.*1998; Spark *et al.*1996). Unbound phages are washed out, however, due to some non-specific binding, phages not specific for the target molecule may also remain bound to the target. Bound phages are then eluted by disrupting the bond between displayed polypeptides and the target. Eluted phages are then used for the infection of host cells for amplification. After several rounds of this cycle, phages with desired polypeptides carrying the desired specificities are analyzed. This cycle is schematically represented in Figure 1.6.
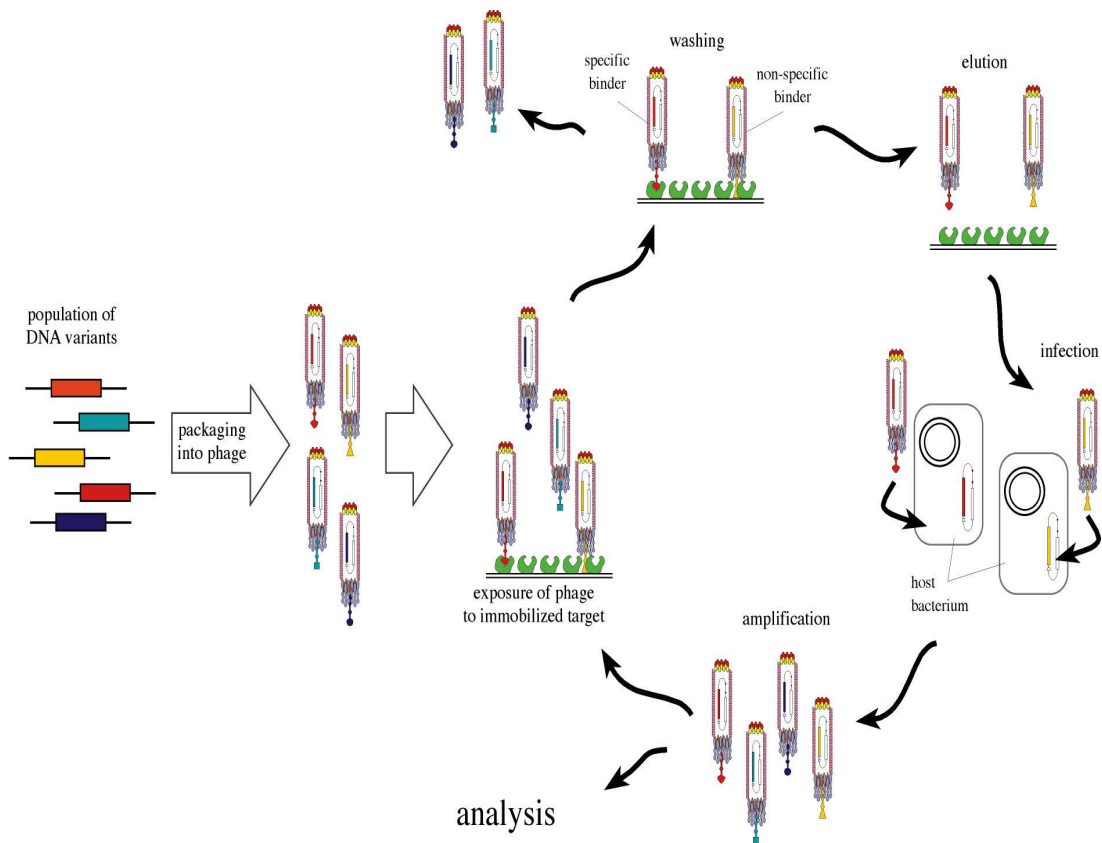


**Figure 1.6:** Phage Display cycle**.**

## 1.1.3.1. Platforms of Phage Display Technology

The earliest phage display systems were based on fusions to either PIII or PVIII. However, this system had some limitations because polypeptides, which are displayed

on the phage were not efficiently displayed and selected (Hufton *et al*., 1999; Sidhu, 2001). In this system, theoretically all the copies of the chosen coat proteins are fusion proteins (Winter et al., 1994). Although this seems to be advantageous, the phage coat protein loses its function and as a result, the phage viability is seriously affected. In return this can lead to the inefficient selection of the desired polypeptides.

This problem was tried to be solved by other approaches. In order to improve phage viability and stability, hybrid phage display system was created. In this system, two copies of the coat protein were employed. One copy was expressed as a fusion protein with the polypeptide of interest and the other in its native form. Therefore, both the fusion and the native coat proteins were exposed on the phage surface. Hence, while the desired protein was displayed on the surface phage, phage viability could be sustained with the presence of the native coat protein making the process efficient, safe and stable (Sidhu, 2001).

Alternatively, phagemid-based hybrid systems were developed (Sidhu, 2000). This approach, which allows the development of larger libraries, differed from phage-based platform in terms of the cloning efficiency (Winter *et al.,* 2000). Phagemid is a plasmid, which contains both phage and plasmid replication origins and one of the phage coat protein genes. The gene encoding the desired protein is fused to the chosen coat protein gene present in the phagemid vector, and then this vector is transferred to *E.coli.* The other phage proteins are provided by a helper phage, which contains a defective replication origin. The resultant phage particles contain wild type proteins from the helper phage, the chosen coat protein fused with the desired polypeptides and the phagemid genome. These platforms were reviewed and illustrated by William, 2002 (Figure 1.7).

Hybrid phage display systems allow the development of many phage display applications and platforms. With this system, although some proteins can be difficult to be displayed because of their properties (e.g. toxicity to *E. coli* or interference with phage production), the size of the protein is not a major factor and the display of most of the proteins should be feasible. Large proteins, up to 40 kDa can be readily displayed on the phage surface (Sidhu, 2001; Hoogenboom *et al.* 1991; Stoop *et al*., 2000).
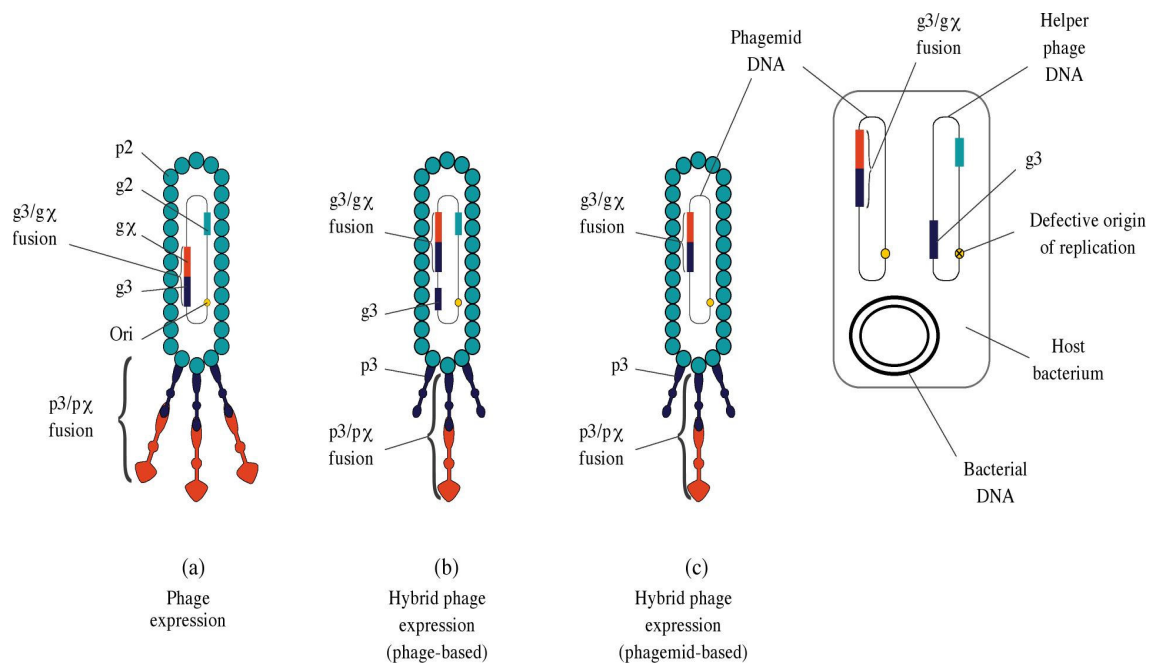
**Figure 1.7:** Platforms of phage display process

## 1.1.3.2. Limitations and Improvement of Phage Display

There had been many attempts to improve phage display since this process was described in 1985(Haek et al., 2003; Sidhu et al., 2003; Hoogenboom et al., 1994)). Despite this process has a lot of advantages, it still comprises some limitations (Azzazy et al., 2002; Willats, 2002). The drawbacks of the earliest phage display approach was tried to be solved by the hybrid phage systems (see, section 1.3.1). Hybrid phage systems considerably improved the phage viability and the continuity of the phage display process.

Although, phagemid based system is preferred to the phage based system due to both its ease of manipulation, both systems suffer from similar drawbacks. Extremely low level display of proteins is a serious problem encountered in both phage and phagemid-based systems (Willats, 2002; Sanghoon *et al*., 2002). The number of cloned fusion proteins displayed on the phage surface is decreased due to the competition between the wild type coat proteins from the helper phage and the cloned fusion proteins from the phagemid during the incorporation into phage particle. Wild type coat proteins are much more efficiently incorporated into the phage particle (Willats, 2002; Winter *et al.* 1994). The number of phage particles without a fusion protein has been

estimated to be up to 99.5% of the total phage population. (Azzazy *et al.,* 2002). Also, phage particles that display the fusion coat protein may carry the helper phage genome instead of the phagemid DNA causing the lost of the selected fusion protein during the amplification step.

Some of the improvements made on the phage display process are focused on the elimination of the phage particles carrying only the wild type coat proteins but not the cloned fusion coat protein to increase the level of display (Willats, 2002). In phagemid-based systems, generally P3 is the chosen fusion partner to the cloned polypeptides because P3 fusions can accommodate larger foreign polypeptides. P3 consists of three distinct domains connected to each other by glycine-rich linker regions. The N-terminal domain (N1) is responsible for viral infection, middle domain (N2) is necessary for binding to the F pilus of the bacterium, and the C- terminal domain is responsible for the viral morphogenesis (Sidhu et al., 2003). P3 fusion proteins are designed in a way that the foreign polypeptide is fused at the N-terminal of P3, since C-terminal fusions are not incorporated into the phage particles (Sidhu et al., 2003). However, since P3 is present in few (5-7) copy numbers on the phage surface, this decreases the probability of the P3 fusion protein to be displayed on the phage surface. In order to circumvent this low probability, P8 protein was used as the fusion partner since P8 is present at considerably high (2700) copy numbers on the phage surface (Sidhu, 2001). However, the presence of more than five foreign amino acids in the P8 fusion protein can prevent the packaging of phage particles leading to reduction in phage production. Coat proteins (P6, P7 and P9) other than P3 and P8 are also used as fusion partners (Table 1.2). However, since they are also present in low copy numbers (5) on the phage surface, they suffer from the same problems encountered with the use of P3. Furthermore the incorporation of their fusion proteins into the phage particles is around 100 times lower compared to P3 fusion proteins.

**Table 1.2.** M13 coat proteins used as fusion partners in phage display

| Protein | Number of amino acids | Molecular weight | Copies per phage | Type of display |
|---------|----------------------|------------------|------------------|-----------------|
| P3 | 406 | 42,500 | –5 | N or C |
| P6 | 112 | 12,300 | –5 | C |
| P7 | 33 | 3,600 | –5 | N |
| P8 | 50 | 5,200 | –2,700 | N or C |
| P9 | 32 | 3,600 | –5 | N |

In phagemid system, the use of a helper phage is necessary to provide all the phage proteins required for phage assembly. However, although the helper phage used in these systems is replication deficient, some helper phage genome replication still occurs at a reduced rate compared to the phage which contains a wild type origin of replication. This low level of helper phage genome replication in turn, may lead to the packaging of helper phage genome rather than the phagemid DNA expressing the recombinant fusion coat proteins displayed on the phage surface (Winter et al., 1994).

There had been some studies towards the elimination of the drawbacks encountered in phage display technology (Kramer *et al*., 2003; Baek *et al*., 2002; Winter *et al.,* 1994; Rondot *et al.,* 2001; Griffiths *et al.,* 1993; McCafferty, 1996; Rondot *et al.,* 2001; Larocca *et al.,* 2001). Although the systems developed during such studies managed to improve the display level of recombinant fusion proteins they could not completely eliminate the loss of affinity selected phages in the total library. As a consequence, these developed systems also need further modifications in order to completely remove the background helper phage particles.

## 1.1.4. Applications of Phage Display Process

Phage display is a powerful technology and it has been applied to a number of different processes and studies (Willats, 2002) Receptor-ligand studies are the most important ones that use this technology (Neri *et al.,* 2000)). Signal transduction research, new drug development and screening, and antibody engineering are other areas where phage display technology is routinely applied proteins and peptides displayed on the phages are represented in Table 3.

### 1.1.4.1. Peptide-Libraries

Phage display of peptides started with the generation of random combinatorial libraries that provide a pool of variants from which peptides with desired characteristics can be isolated by affinity selection (Willats 2002).

Synthetic oligonucleotides with constant lengths and random sequences are generated and then cloned as fusion to one of the coat proteins of M13. In this way, random peptide libraries are constructed and tested by binding to target molecules of interest. PIII or PVIII coat proteins are used for the display of random peptides on M13 filamentous phage (Smith, 1985; Greenwood *et al.,* 1991; Felici *et al.,* 1991).

Peptide sequences identified by phage display have been shown to act as agonists and antagonists of receptors (Doobar et al., 1994). Peptides that neutralize immunoglobulins can be used as diagnostic reagents or therapeutic agents for controlling autoimmune diseases (Blank *et al.*, 1999).

Random peptide libraries can be used for mapping epitopes of monoclonal and polyclonal antibodies to define substrate sites for different enzymes.

Additionally, display of small peptides on phage particles can increase their immunogenicity and their potential as vaccine candidates (Azzazy *et al.*, 2002).

### 1.1.4.2. Phage Display cDNA Libraries

Phage display cDNA libraries enable to identify sequences that are expressed in nature presumably encoding proteins with specific biological functions (Crameri *et al.*, 1996). Other applications involve the specialized libraries constructed using cDNAs for single-chain antibodies (scFv) and Fab antibody fragments (Griffiths *et al.,* 1993; Nissim *et al.*, 1994; McCafferty *et al.,* 1990; Winter *et al.,* 1994).

### 1.1.4.3. Antibody Libraries

Phage displayed recombinant antibodies have a lot of advantages over monoclonal antibodies generated by hybridoma technology. Instead of hybridoma methods that are time consuming and labor intensive for cell screening, antibody genes can be directly from spleen cells using recombinant DNA methods.

Phage displayed antibodies have stable genetic sources. Therefore, Phage antibody technology can also be used to clone and recover monoclonal antibodies from genetically unstable hybridomas. Phage antibody genes can be easily sequenced, mutated and screened to improve antigen binding (Azzazy *et al*., 2002; Holliger *et al*., 1993). Especially, recombinant antibodies have been isolated against toxic antigens or conserved sequences and carbohydrates. Also, antibodies against heavy-chain binding proteins can be obtained which can not be generated by hybridoma technology.

The antibody-fusion proteins provide high sensitivity in diagnostic studies, because they enhance signal amplification after the initial binding of the antibody to its target antigen. Other diagnostic applications are related to the fusion of antibody fragments to alkaline phosphatase, green fluorescent proteins, and lipids.

**Table 3:** Proteins obtained by phage display libraries.

| Protein displayed | Molecular weight (kDa) | Format |
| --- | --- | --- |
| *Secreted proteins* | | |
| Z-domain (Protein A) | 6.5 | pIII |
| Mustard trypsin inhibitor (MTI-2) | 7 | pIII |
| C5a | 8 | pIII (Fos–Jun) |
| Insulin-like growth factor (IGF)-1 | 14.5 | pIII |
| Lipocalins | 17.5 | pIII |
| hGH | 22 | pIII, pVIII, pVIII (C-terminal) |
| Trypsin | 24 | pIII, pVIII |
| Antibody scFv fragments | 25 | pIII, pVIII |
| Subtilisins | 28 | pIII, pVIII |
| Insulin-like growth factor binding protein (IGFBP)-2 | 31 | pIII |
| Peptide–ß2m–MHC complex | 41 | pIII |
| Vascular endothelial growth factor (VEGF) | 2 x 11.5 | pIII |
| Antibody Fab fragments | 2 x 25 | pIII, pVIII |
| Alkaline phosphatase | 2 x 60 | pIII |
| Streptavidin | 4 x 15 | pVIII |
| *Intracellular proteins* | | |
| WW domain | 4 | pIII |
| Src Homology 3 (SH3) domain | 6.5 | pIII |
| FRAP/mTOR FRB domain | 9.5 | pIII |
| Zif268 zinc finger | 10 | pIII |
| Cytochrome $b_{562}$ | 11 | pIII |
| FK506 sinding protein (FKBP)12 | 12 | pIII, T7 display |
| Glutathione S-transferase (GST) | 2 x 25.5 | pIII |

## 1.1.4.4. Phage Enzymes

Several enzymes have been reported to be displayed on M13 bacteriophage to show their catalytic activities. These are alkaline phosphatase, trypsin, β-lactamase (McCafferty *et al.,* 1991; Corey *et al.,* 1993;Siemers *et al.,* 1996). The most common enzyme that has been displayed on phage is penicillin acylase (86 kDa) (Gacio *et al.,* 2003).

Phage display libraries based on suitable enzymes can improve diagnostic by enhancing the stability and catalytic activities of enzymes.

As a result, this multi-purpose tool has allowed the identification of a variety of proteins and peptides that have therapeutic significance. Except the use for therapeutic purposes, there are many applications of the phage displayed proteins and peptides. For example, phage displayed antibodies in plant research can be performed (Willats *et al.,* 2002).

## 1.1.5. Thesis Objectives

In this study, some of the problems of the phage display technology which mainly stem from the use of helper phage were aimed to be solved by the elimination of the use of the helper phage. Although helper phage is necessary for the production of phage particles, it can cause the inefficient display of polypeptides. The helper phage genome can be packaged into the phage particles instead of the phagemid DNA coding for the fusion peptide presented on the phage surface. There is also the possibility of re-infection of cells producing one kind of a fusion protein by the newly produced phage particles carrying other types of fusion proteins with their corresponding phagemid genomes. Both of these events can cause the loss of phage particles carrying the desired fusion peptides during the amplification step following the selection. Therefore, the probability of obtaining the phage particles with desired properties decreases through the amplification cycles. The use of a helper phage also increases the risk of contamination in the laboratories.

For this purpose, cis-acting elements found on the M13 genome responsible for the replication and packaging of the phage particles were deleted from the M13 genome. Then, the M13 genes required for the production of phage particles were aimed to be inserted into the chromosomes of both $F^-$ and $F^+$ *E.coli* strains. By this way, the M13 genes coding for the proteins required for the production of phage particles would be expressed from the bacterial chromosome and there would not be any production of phage particles unless the these bacteria were transformed with a phagemid plasmid DNA containing the M13 origin of replication and the packaging signal.

In the first round of the proposed platform, the fusion coat protein library will be cloned into the phagemid DNA and then transferred into the recombinant $F^-$ *E.coli*

strain by electropoartion. Since F- bacteria do not produce pilli they are resistant to infection with M13 and other filamentous phages. Thus, recombinant F$^-$ *E.coli* cells transformed with a phagemid DNA expressing a particular coat fusion will not be infected by other phage particles produced in other neighboring cells. Hence they will produce phage particles displaying only one kind of a fusion peptide and will only carry the corresponding phagemid DNA.

In the second round, phage particles carrying the coat fusion peptides produced during the first round will be subjected to affinity selection and the selected phage particles with desired properties will then be used for the infection of recombinant F$^+$ *E.coli* strain in order to amplify the phage particles displaying the desired fusion coat polypeptide (figure 1.8).

This phage display platform is a novel approach and if constructed successfully, we believe that it is going to improve the efficiency of this technology considerably.
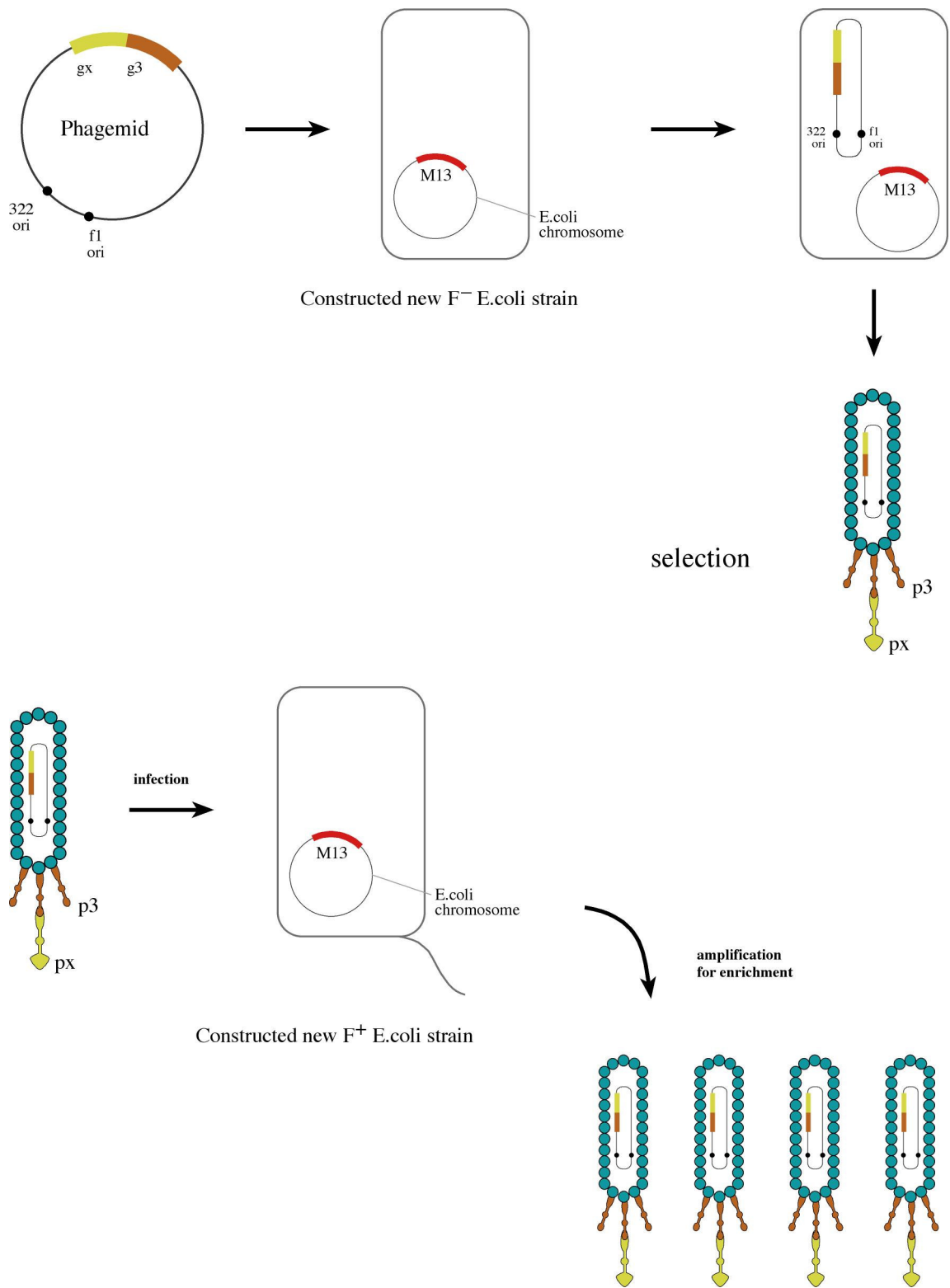
**Figure 1.8:** Procedure of newly created phage display platform

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

Buffers, solutions and suppliers are listed in Appendix C

## 2.2. Methods

## 2.2.1. Bacteriological Techniques

## 2.2.1.1. Bacterial Strains, Plasmids and Media

A list of bacterial strains, plasmids and their sources were presented in Table 2.1 and Table 2.2. respectively. E.*coli* strains were grown in Laura-Bertani (LB) broth at 37°C with continuous agitation.

For the selection of transformed bacteria, LB media containing 1.5 %( w/v) agar and Tetracycline and ampicillin (5µg/ml and 50µg/ml (w/v), respectively) were used.

## 2.2.1.2. Maintenance of Bacterial Strains

Strains were grown on LB agar plates and they were inoculated into LB broth until Log phase. Glycerol stock, 20% in LB broth were prepared and stored at - 80°C for long-term storage.

**Table 2.1:** List of Bacterial Strains

| Strain | Relevant Genotype or Phenotype | Reference |
|---|---|---|
| **XL-1 Blue** | *rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17($r_K^-$,$m_K^+$), *sup*E44, *rel*A1, $\lambda^-$, *lac*⁻, [F' *pro*AB, *lac*I$^q$Z$\Delta$, *M15*, Tn10(Tet$^r$)]. | Bullock et al., 1987 |
| **JM110** | *rps*, (Str$^r$), *thr, leu, thi-1, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44,* $\Delta$ *(lac-proAB),* [F'*tra*D36, *pro*AB, *lac*I$^q$Z$\Delta$ M15]. | Yanisch-Perron et al., 1985 |
| **JM109** | *e14*⁻(*mcr*A⁻), *rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17($r_K^-m_K^-$), *sup*E44, *rel*A1, $\lambda^-$, $\Delta$ (lac-proAB), | Yanisch-Perron et al., 1985 |
| **JM101** | *supE, thi-1,* $\Delta$ (*lac-proAB*), [F' *traD36, proAB,* lacI $^q$ $\Delta$(lacZ) M15] | Yanisch-Perron et al., 1985 |
| **DH5$\alpha$** | F⁻,$\Phi$80$\Delta$ *lacZ*$\Delta$ M15, $\Delta$ (lacZYA-argF),U169. | Woodcock, et al., 1989. |

**Table 2.2:** List of plasmids and phages

| Plasmids | Relevant Genotype or Phenotype | Reference |
|---|---|---|
| **PbluescriptKS+** | *Ap* $^r$; rep (pMB1); F 1 ori; $\Delta$ *lacZ* | Stratagene |
| **Pblu 5'recA** | 5'recA fragment of RecA gene cloned in PbluescriptKS+ | This study |
| **Pblu 5'recA M13** | M13IG⁻ fragment cloned in Pblu 5'recA | This study |
| **PCRII** | *Ap* $^r$; *Km* $^r$ ; F 1 ori; ColE1 ori; $\Delta$ *lacZ* | Invitrogen |
| **PCRII 3'recA** | 3'recA fragment of RecA gene cloned in PCRII | This study |
| **PCRII 3'recA Tet** | *Tet* $^r$ gene cloned in PCII 3'recA | This study |
| **M13 mp19** | $\Delta$ *lacZ* cloned in wild-type M13 bacteriophage | Yanisch-Perron et al., 1985 |

## 2.2.1.3. DNA Isolations

## 2.2.1.3.1. Small-scale Preparation of Plasmid DNA from *E.coli*

After transformation, single colonies were inoculated into 10 ml LB medium supplemented with appropriate antibiotics and incubated with constant agitation for 6-9 h. Plasmid DNA was isolated by alkaline-lysis methods (Sambrook *et al*., 1989) with some minor modifications. One and a half milliliter of each culture were transferred into a 1.5 ml eppendorf tube. The cells were pelleted by centrifugation for 30 s. at 12.000 rpm. Supernant was removed and the pellet was resuspended in 0.1 ml of Solution I and 0.2 ml of freshly prepared Solution II were added. The sample was then mixed gently by inversion several times. Solution III, 0.15 ml, was immediately added. After mixing, each samples were incubated on ice for 3-5 min. Centrifugation was performed at 12.000 rpm for 2 min and supernatants were transferred into fresh tubes. The sample was precipitated with 2 volumes of 99% ethanol at room temperature, and then mixed by vortexing and centrifuged for 5 min at 12.000 rpm. The supernant was removed. The pellet was washed with 1 ml of 70% ethanol and dried in air for 10 min. After drying, DNA was dissolved in 50-microliter $dH_20$ or 1XTE (pH.8) by vortexing briefly. DNA was then kept at $-20°C$ until needed.

## 2.2.1.3.2. Preparation of Bacterial Genomic DNA by CTAB/NaCl

The method of Ausubel *et al.* (1994) was used for genomic DNA isolation.

Strains were grown in LB broth at 37°C overnight with constant agitation. Following morning, 1.5 ml of the culture were transferred into eppendorf tubes and harvested by centrifugation for 5 min at 10.000 rpm. The supernatant was discarded and the pellet was suspended in 567μl 1×TE buffer (Appendix C). Then, 30 μl of 10 % SDS and 3μl of proteinase K (Appendix C) were added. The suspension was mixed thoroughly and incubated for 1 h at 37°C. After this step, 0.1 ml of 5M NaCl was added and mixed well. 80 μl CTAB/NaCl (Appendix C) was added. The samples were mixed and incubated for 10 m at 65 °C. After incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C) was added, mixed gently and centrifuged for 5 m at 10.000 rpm. Aqueous supernatant was transferred into a fresh eppendorf tube. An equal volume of

chloroform/isoamyl alcohol was added, mixed and harvested for 5 min. The supernatant was then transferred into a fresh eppendorf tube and 0.6 volume of isopropanol was added to precipitate the DNA. The samples were shaken until a white DNA precipitate became visible. DNA was precipitated by centrifugation for 5 min. The supernatant was discarded and the pellet was rinsed with 500µl 70 % ethanol. It was centrifuged for 10 min. After this, ethanol was removed. The pelleted DNA was dried and dissolved in 1×TE buffer. Genomic DNA was stored at -20°C.

### 2.2.1.3.3. Large-scale preparation DNA

A Nucleospin maxi preparation kit was used and all the steps were performed according to the manufacturer's instructions.

### 2.2.1.3.4. DNA isolation from agarose gel with Applichem DNA Isolation kit

DNA bands of interest were eluted from agarose gel according to manufacturer's instruction with some minor modifications. After DNA was separated in 0.8% agarose gel at 40mA constant current. DNA bands visualized on u.v.box were excised from the gel with a razor blade under UV light. Three volumes of 6 M NaI solution and glass milk solution from the isolation kit, were added to each of the samples. The samples were incubated for 5 min at 55°C; the contents of the sample tube were mixed and returned to the 55°C for a period of 1-2 m. At this point, it was made sure that gel slice was dissolved completely. The samples were then centrifuged for 30 s at 12.000 rpm. Supernatant was discarded and the pellets were rinsed 3 times with wash solution provided with kit. After rinsing, supernatants were removed completely and the pellets were dissolved in dH$_2$O and DNA were then eluted for 3-5 min at 55°C.

Eluted samples were centrifuged at 12.000 rpm for 1 m. Following centrifugation. Supernatants were carefully transferred into a fresh tube.

## 2.2.1.4. Transformation of Bacterial Cells

## 2.2.1.4.1. Preparation of Competent *E.coli* Cells Using CaCl2 procedure

A single colony, which was freshly grown for 16-20 h at 37°C and inoculated into 100 ml of LB broth in a 1-liter flask, then incubated for overnight. Following incubation, 100 fold dilution was performed and incubated again for ~ 3 h with constant agitation (300 cycles / minute) until an $OD_{600}$ of 0.6 was obtained. For efficient transformation, the number of visible cells should not exceed $10^8$ cells/ml (Sambrook *et al.*, 1989).

After incubation, the culture was transferred into sterile, ice-cold 50 ml falcon tubes, aseptically. Cultures were cooled to 0°C on ice for 10 min. and harvested at 5,000 rpm, for 10 min, at 4°C. The supernatant was removed completely and the pellet was resuspended gently in 10 ml of ice cold 0.1 M $CaCl_2$ (Appendix C), and stored on ice. It was centrifuged again at 5,000 rpm, for 10 min, at 4°C. After discarding the supernatant, the pellet was resuspended in 2 ml ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture. 160µl of competent cell suspension was transferred to a fresh sterile eppendorf tube and added then 40µl of sterile glycerol, mixed gently for storage.

After that, all aliquots were incubated on ice for 4 h. These competent cells were stored at –80°C.

## 2.2.1.4.2. Transformation of *E.coli* Strains with Plasmid DNA

Before transformation, the frozen competent cells were thawed on ice. DNA was added (0.5-1.0µg in a volume of 10µl) into each tube and the contents of the tubes were mixed by swirling gently. The tubes were then incubated on ice for 30 min. After this, the tubes were heated at 42°C for 90 s. Heated tubes were transferred immediately onto an ice bath and cells were chilled for 1-2 min. LB broth, 800µl, were added and the cultures were then incubated for 45 min, at 37°C with constant agitation to allow the bacteria recover and to express the antibiotic resistance marker encoded by the plasmid.

Following incubation, transformed competent cells were plated on LB agar plates containing appropriate antibiotic. Plates were left at room temperature until the

liquid had been absorbed, and the plates were then inverted and incubated at 37°C. Following, colonies were observed in 16 h.

## 2.2.1.4.3. Preparation of Electro-competent *E.coli*

A single colony from a fresh culture was inoculated into 5 ml LB medium and grown overnight with moderate shaking. After overnight incubation, 2.5 ml of the culture were inoculated into 500 ml LB medium in a sterile 2-L flask and grown at 37°C with shaking at 300 rpm to an $OD_{600}$ of ~ 0.5 to 0.7.

The culture was chilled on ice for 10-15 min and was then transferred into a 1- L prechilled centrifuge bottle. Chilled cells were harvested by centrifugation for 20 min at 4,200 rpm at 2°C. All liquid phase was removed completely and the pellet was then resuspended in 5 ml of ice-cold water and mixed well. The cells were immediately centrifuged as above. The supernatant was immediately removed and the pellet was resuspended in10 % 500 µl of ice-cold glycerol, mixed thoroughly. The suspension was stored at –80 °C until use.

## 2.2.1.4.4. Transformation of *E.coli* by Electroporation

Five pico-grams to 0.5 µg plasmid DNA or purified ligation mixture in 1µl was added to tubes containing 40µl of fresh or thawed cells (on ice) and mixed by tapping the tube and the mixture were then transferred immediately into electroporation cuvettes that were prechilled on ice for 5 m. At this point, the volume of DNA added to the cells should have been kept small. Adding DNA up to one-tenth of the cell volume would decrease the transformation efficiency. The cuvette was then placed into the sample chamber.

The electroporation apparatus was set to 2.5 kV, 25µF and the pulse controller was also set to 200 or 400 ohms. Pulse was applied by pushing the button. The cuvette was removed from the chamber and 1 ml of SOC medium was immediately added and the mixture was transferred to a sterile eppendorf tube and incubated at 37°C for 60 m with constant agitation. After the incubation, aliquots of transformation culture were plated on LB agar plates containing appropriate antibiotics.

## 2.2.2. Phage Manipulations

## 2.2.2.1. Preparation of Bacteria

Firstly, bacterial strain carrying an F⁻ pilus episome (JM109-JM107) was streaked onto minimal M9agar plate (Appendix B), incubated for 36 h at 37°C. The genes encoding enzymes involved in proline biosynthesis have been deleted from chromosomes of these bacteria and these are only carried on F-pilus. Therefore, the colonies that can form on this minimal agar plate have F-pilus. A single colony on minimal agar plate was removed and inoculated into 50 ml of LB medium, in a 250 ml flask, agitated for exactly 6 h at 37°C.

 (XL1Blue strain has a tetracycline resistant gene on their F-pilus. Therefore, there is no need to these processes. If colonies form on LB agar containing tetracycline, these colonies carry F-pilus. ).

## 2.2.2.2. Infection with M13

A plaque of bacteriophage M13 derives from infection of a single bacterium by a single phage particle. The progeny particles infect neighboring bacteria, which in turn release another generation of progeny. If the bacteria grow in semisolid medium (containing, e.g., agar), the diffusion of the progeny particles is limited.

M13 does not kill the cells they infect but make their growth slow nearly two-fold. Under these circumstances, a plaque forms as an expanding zone of slowly growing bacteria that becomes visible to the naked eye against a background of more turbid bacterial growth within the agar overlay.

Sterile tubes containing 5 ml of melted top agar (made up in LB medium) were prepared and stored at 47°C. Ten-fold serial dilutions of bacteriophage stocks in LB medium were prepared. These dilutions were then added into cultures that were prepared before (XL1Blue or JM109) for infection, mixed gently and incubated at 37°C without agitation for infection. After that, 100µl of infected culture were added to each tube containing melted top agar, then mixed by vortexing gently. Forty microliters of X-Gal solution (Appendix C) and 4µl of a solution of IPTG (Appendix C) were immediately added and mixed by manually. The contents of tubes were poured onto

plates containing LB agar and placed at room temperature to allow the top agar to harden for 5 min. Then, the plates were incubated at 37°C for 12 h. Blue plaques were begun to appear after 4 h. of incubation.

### 2.2.2.3. Picking Bacteriophage M13 Plaques

One milliliter of LB medium was dispensed into a sterile tube. A plaque was transferred using a sterile stick, into this medium. The suspension was incubated for 2 h at room temperature to allow the bacteriophage particles to diffuse out of the agar.

The suspensions of bacteriophage particles were stored in LB medium at −20 °C to prevent the loss of viability.

### 2.2.2.4. Preparing Stocks of Bacteriophage M13 from Single Plaque

50 ml of a culture of a F$^+$ *E.coli* strain (XL1Blue or JM 109) were prepared and dispensed into a sterile culture tube containing 2 ml of LB and one tenth of the suspension of bacteriophage particles derived from single plaque (Section 2.2.2.3.)

The infected culture was incubated for 4 h at 37°C with constant agitation. To minimize the possibility of selecting deletion mutants, cultures infected with recombinant bacteriophage M13 were not grown longer than 5 h.

The infected culture was transferred into a sterile eppendorf tube and centrifuged at 12,000 rpm for 5 min at room temperature. The supernatant was transferred into a fresh eppendorf tube. The stocks were stored at 4°C or −20°C without loss of infectivity.

The remaining 1 ml of infected bacteria culture was used to prepare single stranded or double stranded replicative form of bacteriophage DNA.

### 2.2.2.5. Preparation of Bacteriophage M13 DNA

Bacteria, which were infected with bacteriophage M13, contain the double stranded replicative form of bacteriophage M13 DNA and produce virus particles containing single stranded progeny DNA (Sambrook et al.,1989)).

## 2.2.2.5.1. Small-scale Preparation of Replicative Form (RF) Bacteriophage M13

The method described by Current Protocol (1999) was used for the small-scale preparation of Replicative Form (RF) of Bacteriophage M13 with some minor modifications.

An infected culture (2 ml) was prepared as described before on page 24.1.2-1.5 ml of the infected culture was transferred into a fresh eppendorf tube and harvested by centrifugation at 12,000 rpm. for 5 m at room temperature. The infected bacteria formed a visible pellet and the bacteriophage particles remained in the supernatant.

The supernatant was transferred into a fresh, sterile eppendorf tube for using preparation of single stranded bacteriophage DNA at a later stage. Remaining of the supernatant was then discarded carefully and the bacterial pellet was resuspended in 100µl of ice-cold Solution I (Appendix C) by vortexing. After this, 200µl of freshly prepared Solution II at room temperature (Appendix C) was added and the contents were mixed by inverting the tube rapidly several times. The tube was then placed on ice and 150µl of ice-cold Solution III (Appendix C) was immediately added and mixed by inverting the tube for several times. The tubes were centrifuged at 12,000 rpm for 2 min. The supernatant was transferred into a fresh tube and the RF DNA was extracted and precipitated using Applichem DNA Isolation Kit according to manufacturer's instruction.

## 2.2.2.5.2. Small-scale Preparations of Single-stranded Bacteriophage M13 DNA

1.0-1.2 ml of the supernatant which was prepared and stored at 4°C were transferred to a fresh eppendorf tube, and 200µl of a solution of 20% PEG 8000(Appendix C) in 0.3 M NaCl was added and the content was mixed by inverting the tube for several times. The sample was incubated for 5 min at room temperature, and then centrifuged at 12,000 rpm for 5 m at 4°C.

Supernatant was removed carefully and the pellet was resuspended in 100µl of TE (pH 8) by vortexing. For the extraction of DNA, 200µl of phenol equilibrated with Tris-HCl (pH 8) (Appendix C) was added and mixed well. The sample was incubated at

room temperature for 2 m. and mixed by vortexing and centrifuged at 12,000 rpm for 1 min at room temperature. The upper phase was transferred into a fresh tube containing 300µl of 25:1 absolute ethanol: 3M NaAc (pH 5.2) solution (Appendix C), mixed gently and incubated at room temperature for 15 min.

After the incubation, precipitated single stranded bacteriophage M13 DNA was recovered by centrifugation at 12,000 rpm. for 10 m at 4°C. The supernatant was removed gently. 200µl of 70% ethanol at room temperature was then added and mixed briefly. The sample was centrifuged. The supernatant was immediately removed and the pellet was placed at room temperature for 10 m to allow any residual ethanol to evaporate.

The pelleted DNA was dissolved in 50µl of TE (pH 8) and prepared single stranded bacteriophage DNA was stored at –20 °C.

## 2.2.2.6. Transfection with Bacteriophage

## 2.2.2.6.1. Preparation of Competent Bacteria for Transfection

The method described by Sambrook et al., 1989 was used for the preparation of competent cells.

A master culture of bacterial strain JM109 was streaked onto a minimal M9 agar plate. (A master culture of bacterial strain XL1-Blue was also streaked onto LB-agar plate containing tetracycline. Frozen competent bacteria were prepared as described in section 2.2.1.4.1.

## 2.2.2.6.2. Transfection of Competent Bacteria with Bacteriophage M13

The method described by Sambrook et al., 1989 was used for the transfection of competent cells with some modifications and optimizations.

An overnight culture of bacteria (as described in section 2.2.2.1.) was prepared in LB-medium at 37°C with constant shaking. Following incubation, a frozen competent XL1-Blue or JM109 at -70 °C was removed and thawed at room temperature, then placed on ice for 10 m. After this, 50µl of the competent bacteria was transferred into each of a series of sterile tubes. Five micro liters of each of the ligation reactions was

added into these tubes. The DNAs and bacteria were mixed thoroughly. After this, these tubes were incubated on ice for 30 min.

A set of sterile culture tube containing 5 ml of melted LB-top agar was prepared and stored at 47°C before use. The tubes containing the competent **bacteria and DNA** were incubated at 42°C for 90 sec. The tubes were transferred immediately onto an ice bath and incubated for 2 min. Following incubation, 175µl of LB-broth were added. The tubes were mixed gently and at the same time 40µl of a solution X-gal and 4µl of a solution IPTG were added into the tubes containing melted LB-top agar, and mixed by vortexing. The transfected bacteria were transferred into the tubes containing melted LB-top agar and 200µl of overnight culture of XL1-Blue or JM109, were immediately added and mixed by vortexing. After this, the mixtures, which were prepared before, were poured into LB-agar plates. The plates were incubated at room temperature for 5 m to allow the top agars harden. Then the plates were inverted and incubated at 37°C. Plaques begun to appear after 4 h. and developed fully after 8-12 h of incubation.

Plaques formed by wild type bacteriophage M13 were deep blue while recombinants were colorless.

## 2.2.2.7. Plaque Formation Assay

Plaque formation assay was performed by combining two microbiological techniques (Sambrook *et al*., 1989; G.Soltes *et al*., 2003).

XL1Blue and infected culture were grown in liquid media (2X YT+1% (w/v) glucose) for overnight and following morning, XL1Blue overnight culture was diluted and grown to midlog phase ($A_{600}$ of 0.6-0.8). Infected bacterial culture was heat killed at 65 $^0$C for 10 m and the culture was harvested by centrifugation for 10 m at 4000 rpm. Following centrifugation, supernatant was aliquoted without further purification and stored at –20 $^0$C.

After that, for plaque formation assay, XL1Blue was infected with obtained phage particles at 37 $^0$C for 30 m. The serial dilutions of infected culture were performed and diluted infected cultures were mixed with 3 ml of melted 2X YT soft agar (Appendix B), and spread on plate containing 2X YT agar (Appendix B). After overnight incubation, the number of plaques was determined.

## 2.2.3. DNA Manipulation Techniques

### 2.2.3.1. Digestion of DNA with Restriction Enzymes

All restriction enzymes were purchased from Fermentas and the digestions were achieved according to the manufacturer's instructions.

All reactions were prepared using concentrated buffers supplied with the enzymes.

### 2.2.3.2. Dephosphorylation of DNA

To prevent self-ligation of vector DNA ends in cloning experiments, 5' termini of vector DNA was dephosphorylated by using Calf Alkaline Intestinal Phosphatase (CIAP) (Fermentas).

Five micro liters of phosphatase buffer (supplied with the enzyme), 14µl $dH_20$ and 1 U CIAP was added to 30 µl of reaction mixture containing 1.5 µg linear plasmid DNA digested with restriction endonuclease(s).

Dephosphorylation reaction was achieved at 37°C for 30 m. To stop the reaction, the mixture was incubated at 85°C for 15 m. DNA was then purified by phenol/chloroform extraction.

### 2.2.3.3. DNA Ligation Reactions

To join double strand DNA digested with restriction endonuclease(s), T4 DNA ligase (Fermentas) was used according to the manufacturer's instruction with some modifications.

All reactions were carried out at 22°C for overnight.

## 2.2.4. Purification and Extraction of DNA Fragments

### 2.2.4.1. Phenol-chloroform Extraction

Phenol-chloroform extraction was used to remove any remaining cellular debris and other substances from cell lysates and to remove any restriction or modification enzymes and buffers after a reaction.

An equal volume of phenol added to the lysate or DNA solution and mixed thoroughly and incubated on ice for 10 min and then the suspension was centrifuged at 10,000 rpm for 2 m. The upper phase was transferred into a fresh tube and equal volume of phenol and chloroform were then added and mixed thoroughly. Centrifugation was performed at 10,000 rpm for 2 m. The resulting upper phase was carefully transferred into a fresh tube and mixed with an equal volume of chloroform and centrifuged. After this, aqueous phase was transferred into new tube containing 1/10 sample volume of 3M NaAc (pH5.2)(Appendix C) and mixed. Two and a half sample volume of 99% ethanol was added and mixed well. The sample was then centrifuged for 15 min at 7,000 rpm. Liquid phase was discarded and DNA pellet was washed with 70% ethanol. After centrifugation for 5 m. at 7,000rpm, ethanol was discarded.

The pellet was dried at room temperature and was then dissolved in 1×TE buffer (Appendix C).

### 2.2.4.2. Ethanol Precipitation of DNA

In order to recover DNA from aqueous solutions or to concentrate the DNA in a solution, the DNA was precipitated by adding 1/10 volume of 3M NaAc (pH5.2) and 2.5 volume of cold ethanol (stored at -20°C), and the sample was then incubated at –80°C for 30 m. After the incubation, the mixture was centrifuged for 5 min at 12,000 rpm and the pellet was dissolved in a desired volume of 1×TE (pH 8) or dH$_2$0.

### 2.2.4.3. Purification of DNA by Applichem DNA Isolation Kit

All the steps were performed according to the manufacturer's instructions.

## 2.2.4.4. Separation of DNA Molecules by Agarose Gel Electrophoresis

Agarose gels (0.8 or 1%)were prepared by melting the agarose in 1X TAE buffer. The melted solution was then poured into a horizontal gel apparatus and allowed to harden. The gel was covered with 1×TAE and the samples were mixed with the gel-loading buffer and the mixtures were then pipetted into the wells formed by the removal of the comb after the gel hardened. To achieve the movement of the DNA molecules, an electric field of 40 mA was applied until Bromophenol blue reached the half of the gel. DNA fragments were visualized in UV box.

## 2.2.5. Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a method for amplification of selected nucleic acids sequences *in vitro* (Mullis and Faloona, 1987). The method consists of repetitive cycles of DNA denaturation, primer annealing, and extension by DNA polymerase (Mullis *et al.*, 1986).

## 2.2.5.1. Primers

The sequences of primers, which were used for PCR, are given in Appendix D.

# CHAPTER III

# RESULTS

## 3.1. Homologous Recombination

There are several methods designed for the replacement, deletion or mutation of desired chromosomal sequences in bacteria. Two of these methods, which can also be used for the specific integration of DNA sequences into the chromosome, are non-replicative plasmid mediated insertions (Leenhouts *et al*., 1989) and temperature sensitive (ts) replicative plasmid mediated insertions (Biswas *et al*., 1993).

For several organisms it has been shown that a plasmid that is unable to replicate in the recipient strain can integrate efficiently into the host chromosome when it carries homologous chromosomal sequences. The region of homology provided usually stimulates the integration of the plasmid DNA by a Campbell-like mechanism (Fig. 3.1 a). In the presence of two separate chromosomal sequences on such a plasmid the recombination event leading to the Campbell-like integration in one of the sites, may be followed by another recombination event in the other site (Fig. 3.1 b), which, in turn, may result in the replacement of the chromosomal segment between the two sites. There are two possible sites for a recombination event after the first one. Assuming equivalent sizes of the cloned DNA, 50% replacement and 50% reversion to wild-type (original) will take place (Fig. 3.1 c). In order to achieve the insertion of the M13 coding regions and the deletion of the *recA* gene by homologous recombination, the flanking DNA regions (each of approximately 1.2 kb) adjacent to the *recA* gene were cloned in a recombination cessette in the same orientation as in the chromosome of *E.coli*.
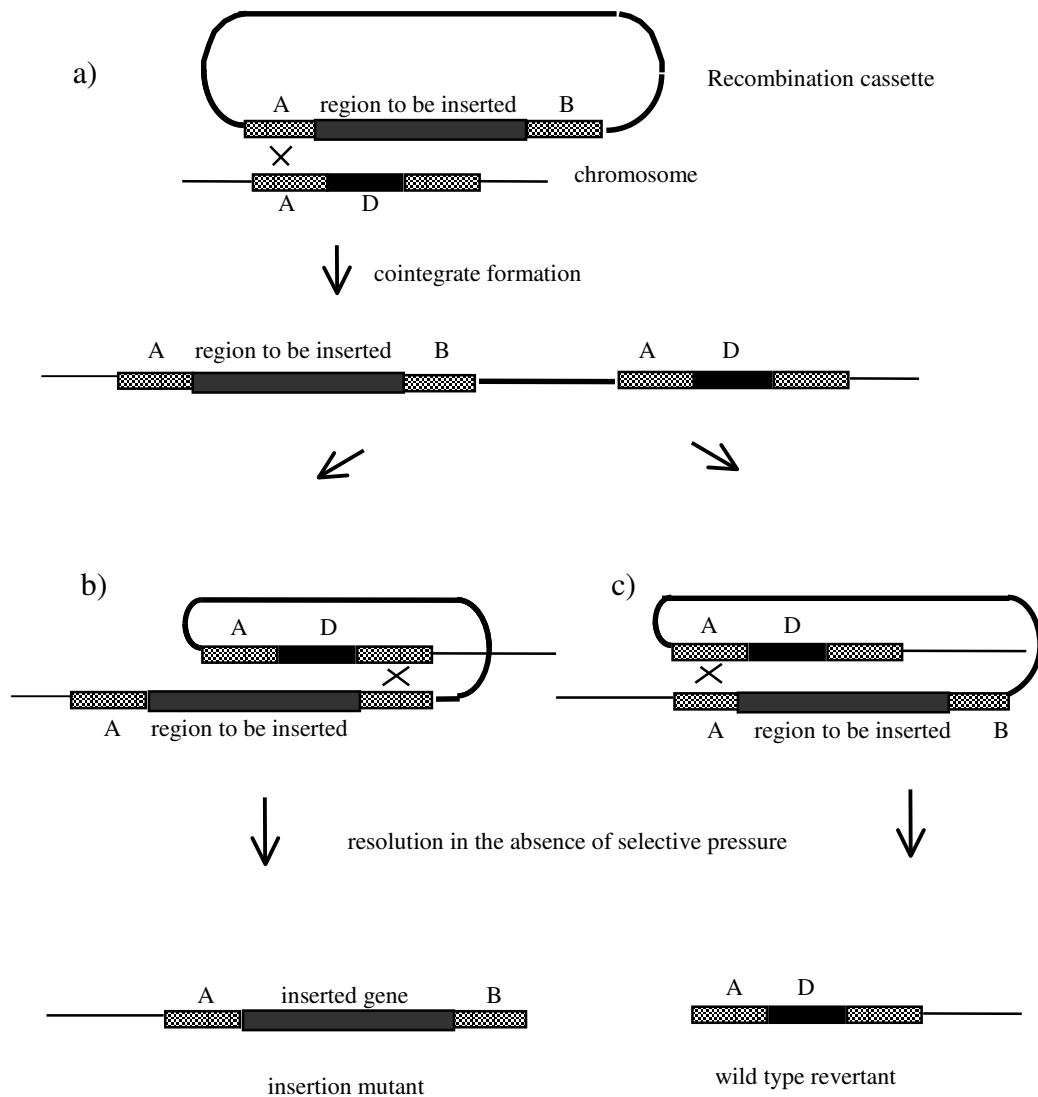
**Figure3.1:** General strategy for the gene insertion method. The initial recombination event occurs between homologous sequences of the cassette and the chromosome and results in the formation of a cointegrate (a). Cointegrates are identified by plating transformed cells onto medium that selects for the antibiotic resistance gene encoded by the cassette (eg. tetracyclin resistance). After growth on non-selective medium, the cointegrates undergo a second recombination event, excising the cassette from the chromosome. Depending on the site of the second recombination event, the gene D on the chromosome will either be replaced by the region to be inserted (b) or remain in the chromosome (c).

## 3.2. Strategy used for the Integration of M13 genome without the IG region (M13ΔIG) into the *E.coli* Chromosome by Homologous recombination.

Prior to the integration of the M13ΔIG genome into the E. coli chromosome, a locus on the chromosome was chosen as the target for integration. We chose this locus to be the recA gene of E. coli because recA protein plays a major role in the cell in DNA repair by recombination. Thus, recA+ cells are expected to be more amenable to homologous recombination. Also, the consequences of insertion of the target DNA into the recA locus by homologues recombination would result in the loss of recA gene which would make the constructed new strain recA-. This is advantageous since recA-E. coli strains are more suitable in cloning experiments due to the deficiency of recA-strains in recombination events.

Thus, in order to integrate the M13ΔIG, a recombination cassette was designed. Ultimately this cassette would lack an active replication origin of any sort in E. coli prior to the transfer of the cassette in to E. coli cells, so that any colonies expressing the marker gene present on the cassette would have to be the ones where the recombination cassette integrated into the bacterial chromosome. This cassette would contain the 5' and the 3' flanking regions of the E. coli recA gene which would provide homology required for the homologous recombination event and the M13ΔIG DNA in between these two flanking regions. There would also be a selectable marker gene which was decided to be the tetracycline resistance gene.

## 3.3. Construction of the Recombination Cassette

Primers P55 and P53 (with *Pst*I and *EcoR*I 5' extensions on P55 and P53 respectively) were designed for the production of 1.3 kb.fragments upstream of recA gene (figure 3.2). This fragment was called "5'recA". *E.coli* chromosomal DNA was used as the template and the reaction conditions were as follows:

Step1: 94°C for 1 min.
Step2: 94°C for 1 min. (Denaturation)
Step3: 54°C for 2 min. (Annealing)
Step4: 72°C for 1 min. (Elongation)

Step5: 72°C for 10 min. (Final extension)

30 amplification cycles

This fragment was digested with EcoRI and PstI restriction enzymes and then ligated into the MCS of the plasmid pBluescript KS+ which was also digested with the same enzymes. After the transformation of compotent *E.coli* cells with the ligation mixture, transformant colonies were selected on LB-agar plates containing ampicillin, X-gal and IPTG . Plasmid DNA were prepared from selected white colonies and they were checked for the presence of 5'rec A fragment by restriction enzyme digestion analysis. One of the plasmids which was confirmed to contain the 5'rec A fragment was used for further experiments. This plasmid was called "pBlue-5'recA" (figure 3.3).

Primers P35 and P33 (with *Sac*I and *Xho*I extensions respectively) were designed for the amplification of the 1.2 kb. fragment which was called "3'recA" immediately downstream of the recA gene. The reaction conditions for this amplification was same as for the amplification of 5'recA fragment. This fragment was then digested with *Xho*I and *Sac*I restriction enzymes and cloned into the MCS of the plasmid PCRII which was also digested with the same enzymes. After the transformation of compotent *E.coli* cells with the ligation mixture, transformant colonies were selected on LB-agar plates containing ampicillin. Plasmid DNA was prepared from ampicillin resistant colonies and they were checked for the presence of the 3'recA fragment by restriction enzyme digestion analysis. One of the plasmids which was confirmed to contain the 3'recA fragment, called "pCRII-3'recA", was used for further experiments (figure 3.4).
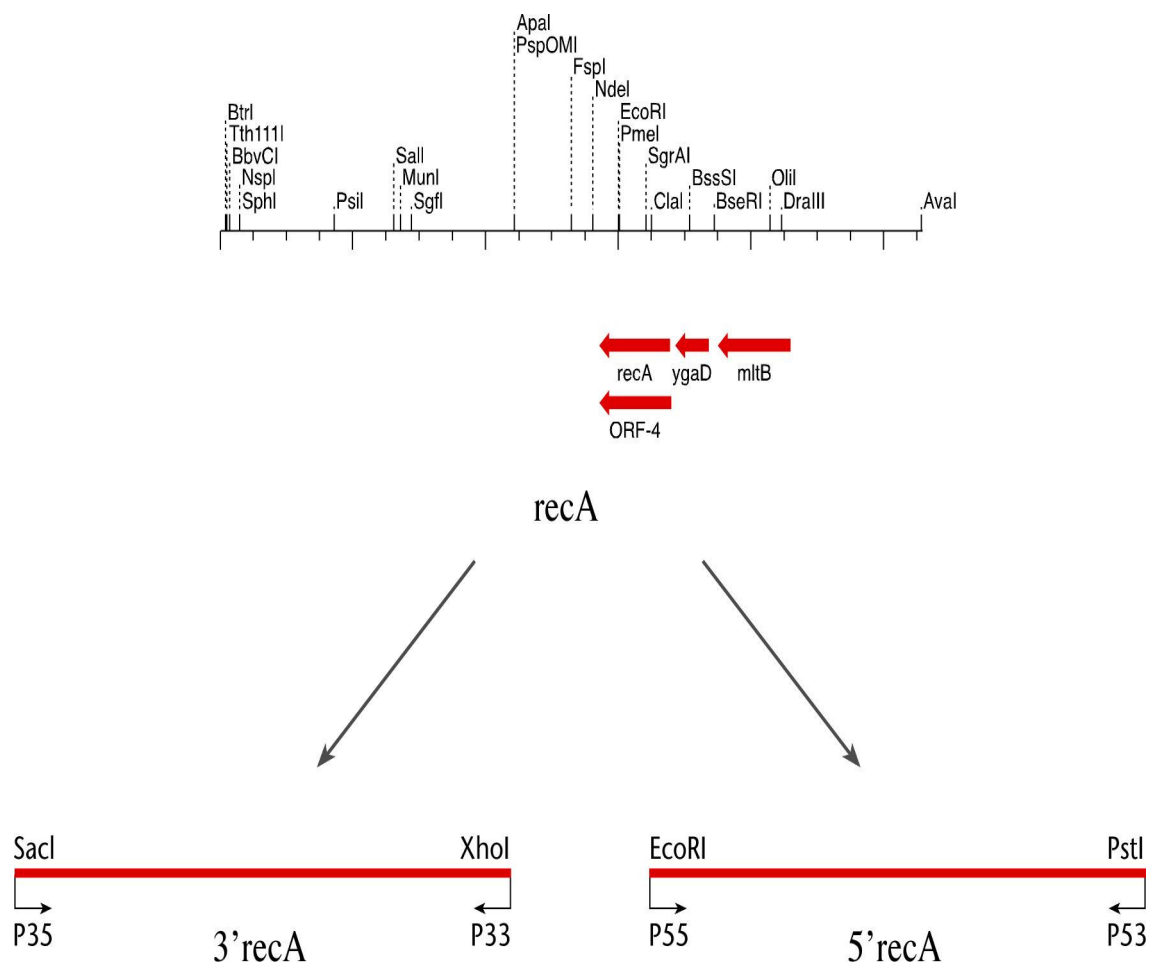
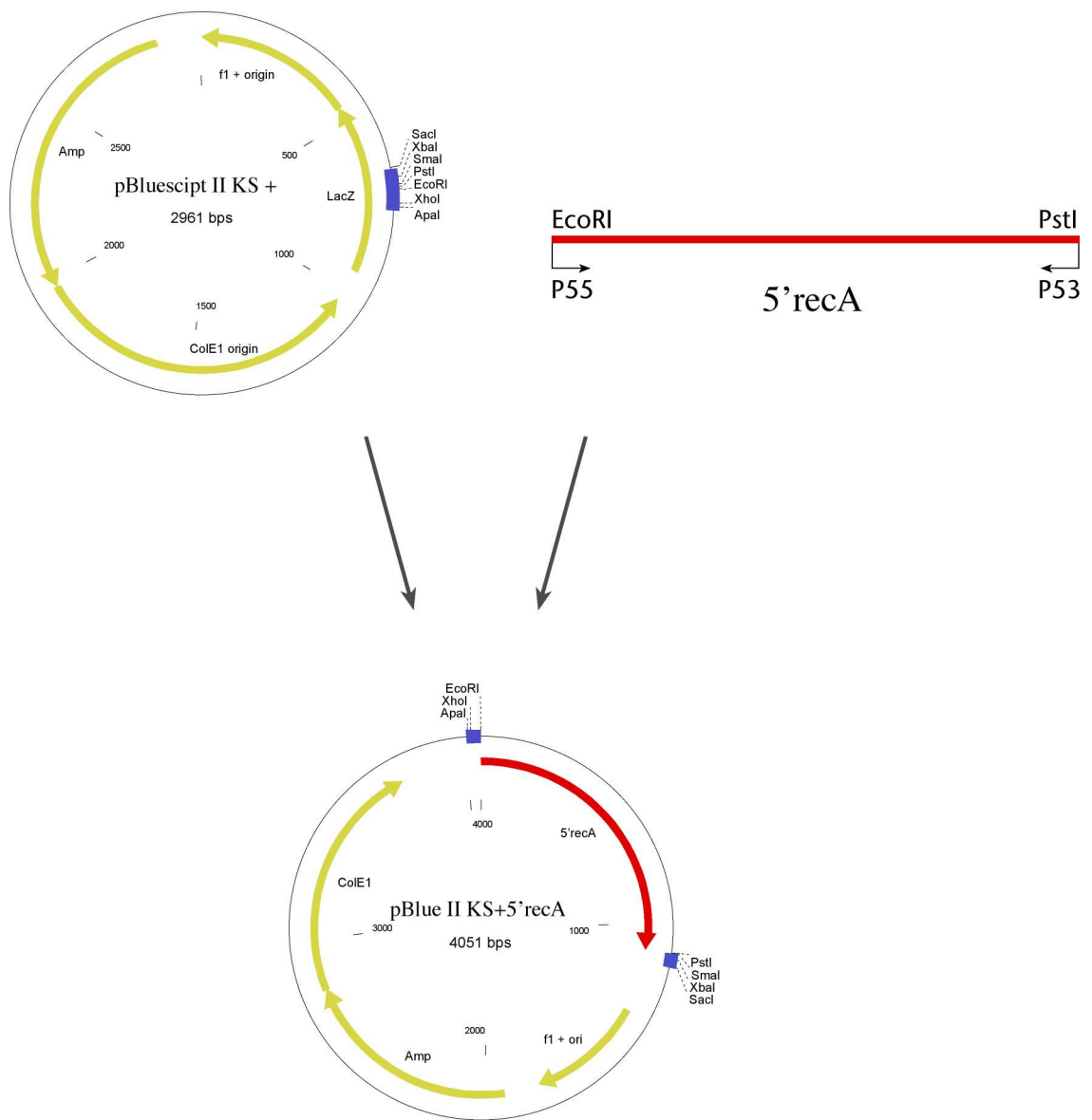**Figure 3. 2:** Amplifications of downstream and upstream fragments of the RecA gene.

**F**igure  3. 3: Construction of Pblu 5'recA vector
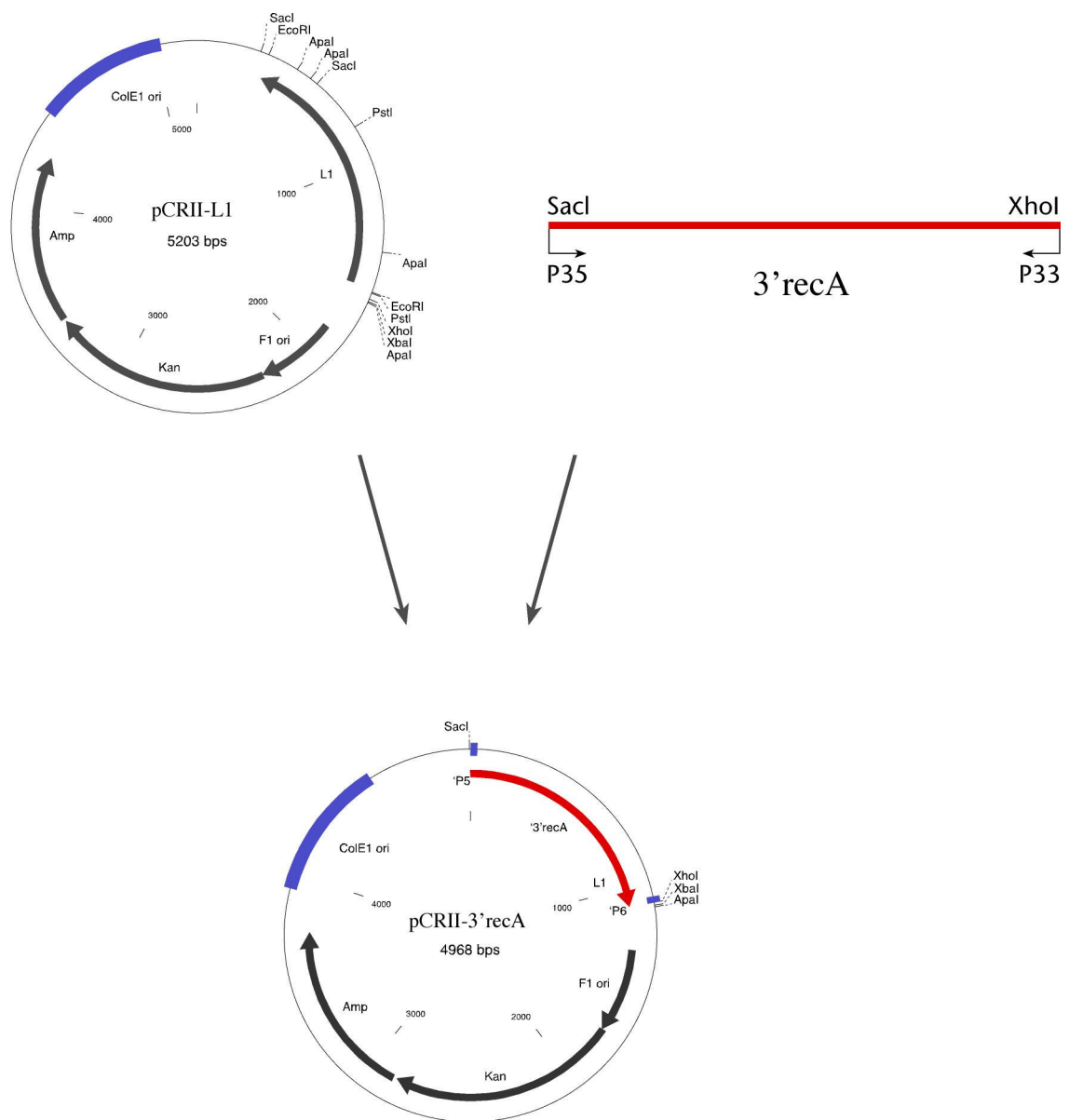
**Figure 3. 4:** Construction of PCRII 3'recA vector

In order to clone the marker gene (tetracycline resistance gene), primers T3 and T5 with *Xho*I and *Xba*I extension respectively were designed for the amplification of the tetracycline resistant gene (*tet*) from the plasmid pBR322 (figure 3.5). The *tet* gene was used in order to monitor the integration of M13 into *E.coli* genome. Both the *tet* gene and the pCRII 3'recA plasmid described above were digested with *Xho*I and *Xba*I and were then ligated to each other to produce the plasmid PCRII 3'recA-Tet (Fig. 3. 6)

Primers M5 and M3 with *EcoR*I and *Sac*I extension, respectively were also designed for the amplification of a 6,072 bp fragment on the M13 genome. This

fragment (M13ΔIG) contains all of the bacteriophage M13 protein coding regions (geneI, geneII, geneIII, geneIV, geneV, geneVI, geneVII, geneVIII, geneIX and geneX) except the intergenic region (IG) which contains the origin of replication and the packaging signal for the phage genome (figure 3.7.). M13ΔIG and the plasmid Pblu5'recA were both digested with *Sac*I and *EcoR*I enzymes and ligated to to each other to obtain the plasmid pBlue5'recA M13 (Fig. 3. 8.).

pBluescriptKS+ vector carries a plasmid origin of replication and the IG region of the phage M13. Since plasmids carrying the M13 origin of replication can be packaged into M13 phage particles, such plasmids are also called as phagemids. For this reason, we decided to use pBluescriptKS+ in order to be able to confirm the functionality of the M13 genes present in the M13ΔIG fragment. Since M13ΔIG lacks the replication origin of M13, M13ΔIG cloned into the phagemid pBluescriptKS+ would have all the necessary sequences for the production of phage particles. Thus this construct would be able to produce phage particles in the bacteria it is transformed with and would give rise to plaque formations in a plaque assay confirming the functionality of the phage genes present on the PCR amplified M13ΔIG fragment.

Plasmid DNA from the ampicillin resistant colonies were analysed by restriction enzyme digestion and one of the colonies which was confirmed to contain the plasmid pBlu5'recA M13 was further subjected to plaque assay analysis in order to confirm the functionality of the cloned M13ΔIG DNA (figure 3.9).

After the observation of plaque formation, both the 5'recA and the M13ΔIG sequences present on the pBlu5'recA M13 plasmid, were planned to be transferred into another plasmid vector (pUC18) which does not contain a bacteriophage replication origin, in order to construct the backbone for the homologous recombination cassette.
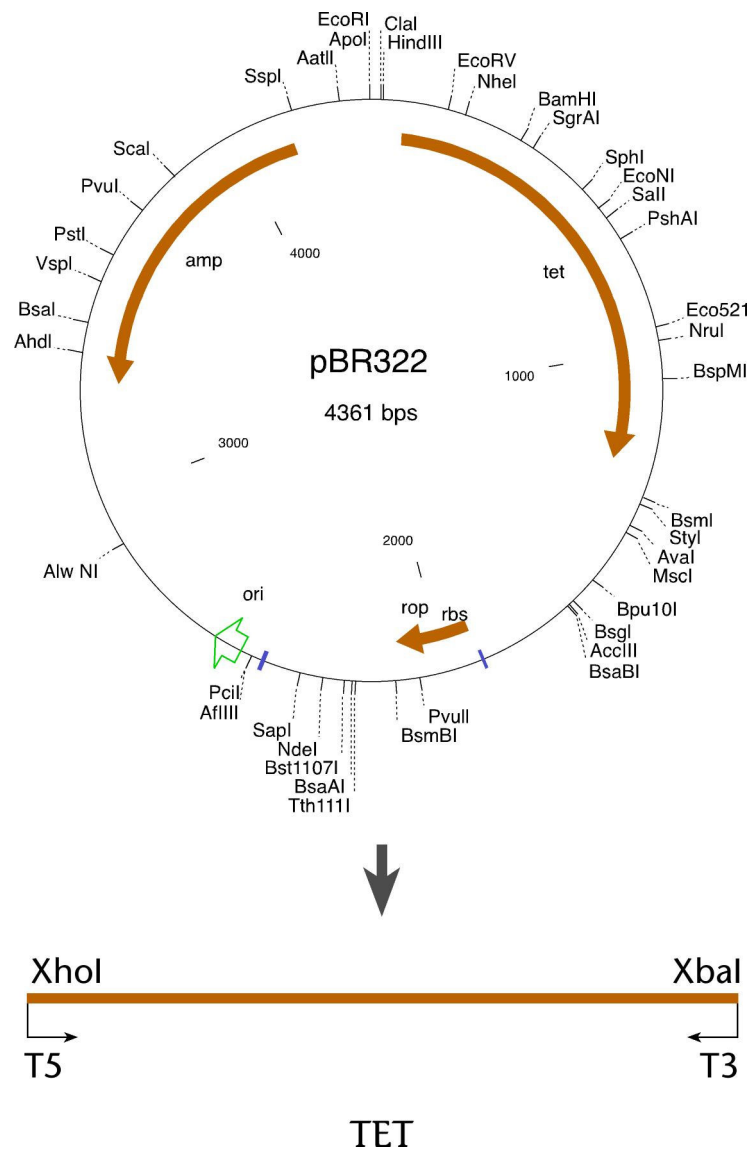
**Figure. 3. 5:** Amplification of Tet gene
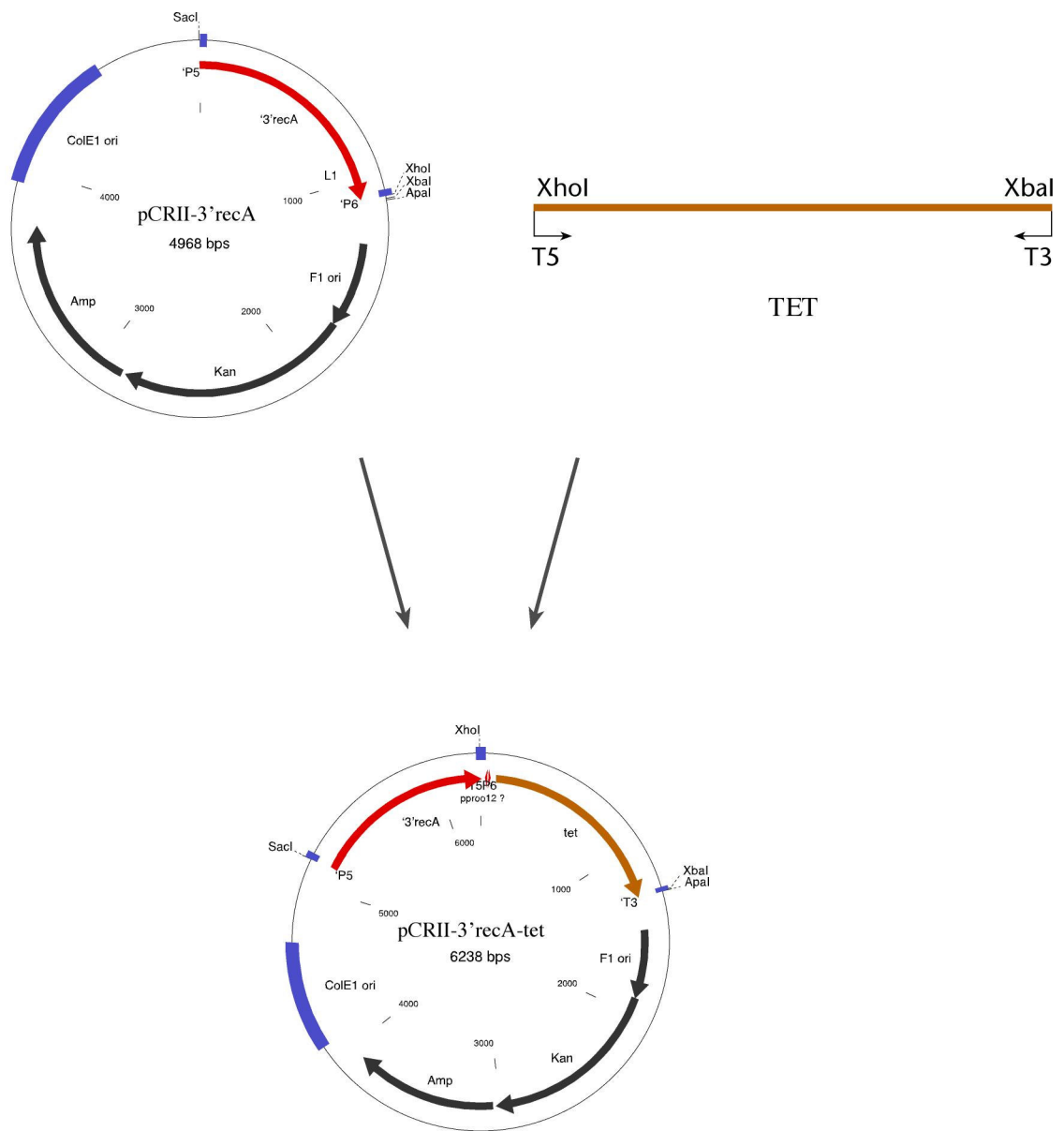
**Figure 3. 6:** Construction of PCRII 3'recA Tet vecto*r*
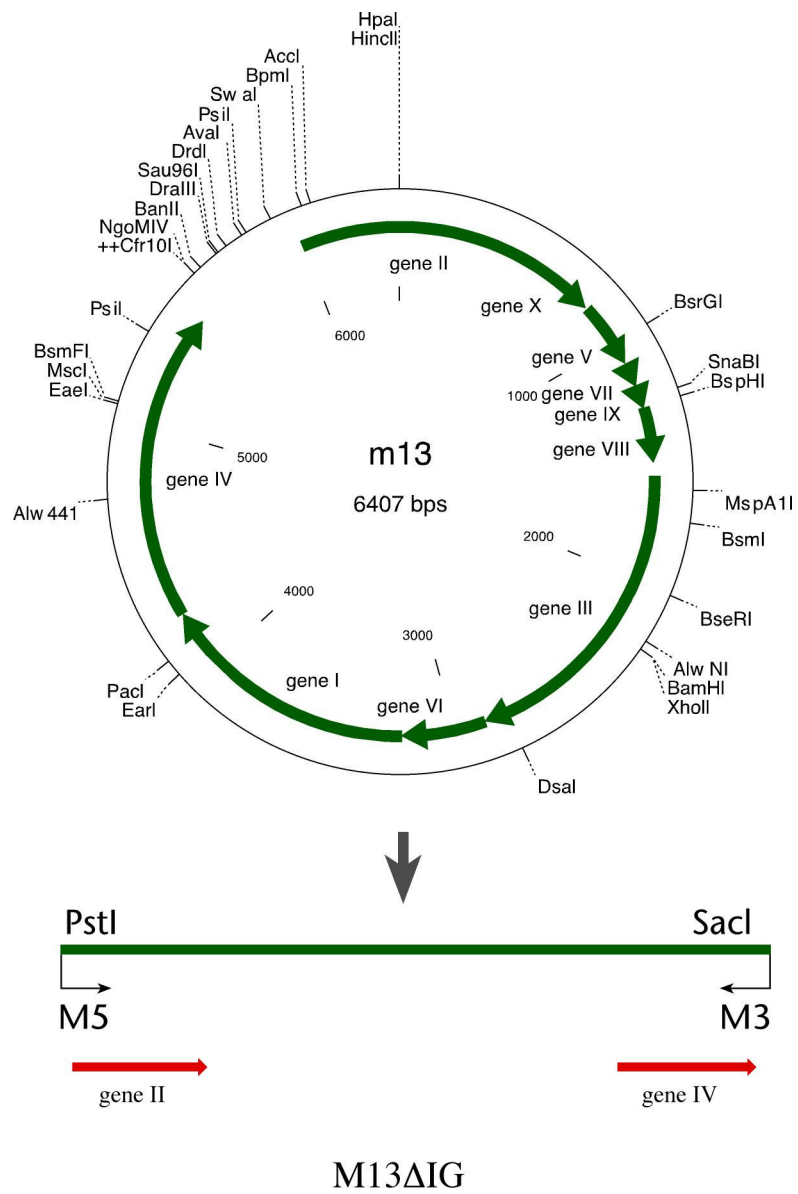
**Figure 3. 7:** Amplification of M13 genes excluding IG region
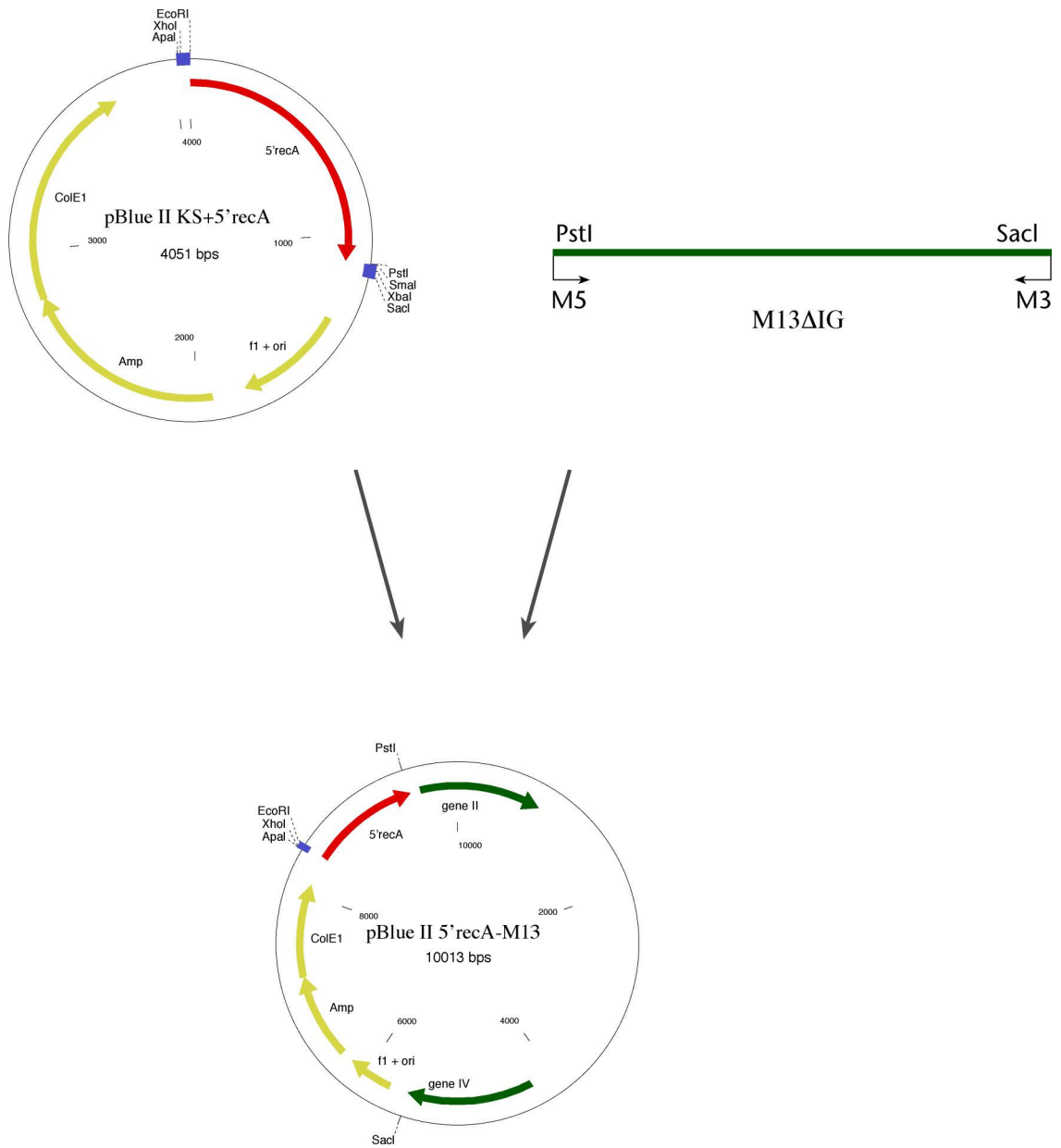
**Figure 3. 8:** Construction of Pblu 5'recA m13 vector
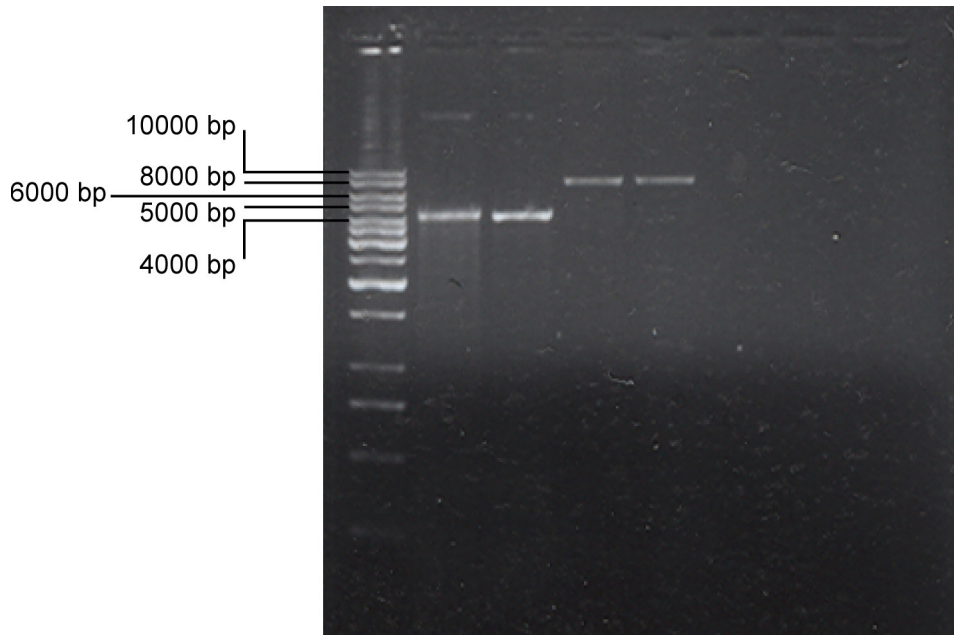
**Figure 3.9:** Confirmation of the location of additional EcoRI site. Lanes 1. 1 kb DNAmarker; 2. Pblu 5'recA digested with EcoRI; 3. Second control of Pblu 5'recA digestion; 4. M13mp19 digested with EcoRI; 4. Second control of M13mp19.
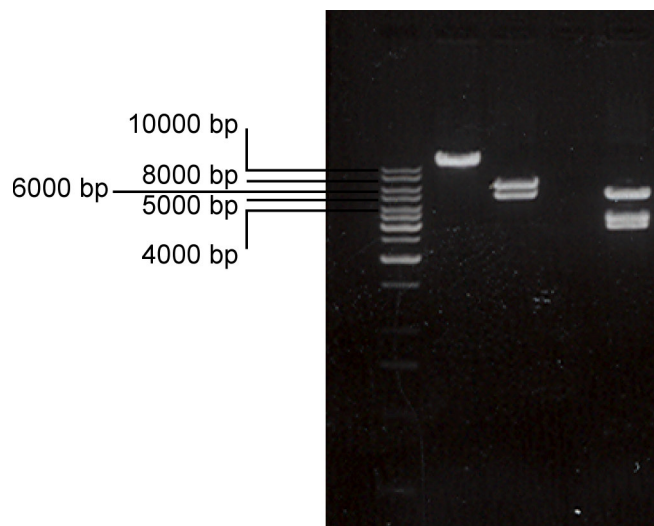


**Figure 3.10:** Restriction digestion analyses of Pblu 5'recA M13. Lanes 1. 1 kb DNAmarker; 2. Pblu 5'recA m13 digested with SacI; 3Pblu 5'recA m13 digested with EcoRI; 5. Pblu 5'recA m13 digested with EcoRI-SacI.

For this purpose, both the pBlu5'recA M13 and the pUC18 plasmids were digested with *EcoR*I and *Sac*I. . According to the sequence data of the plasmid pBlu5'recA M13, digestion of this plasmid by *EcoR*I and *Sac*I enzymes should produce two fragments with sizes of around 7.1 and 2,9kb. However, upon analysis of the digested fragments by agarose gel electrophoresis, three fragments of sizes 4.4, 2.7 and 2,9kb were observed (fig. 3.10). Further digestion analysis of this plasmid and the M13 genomic DNA with other restriction enzyme combinations (PstI, EcoRI and PstI-EcoRI) confirmed that there was an extra EcoRI site generated at the gene III region of the M13ΔIG fragment (data not shown), indicating the fact that a mutation was introduced into the plasmid pBlu5'recA M13 most probably during the PCR amplification of the M13ΔIG fragment.

After this finding the plaque formation assay was repeated for E. coli cells that harboured the plasmid pBlu5'recA M13 and the results were compared with the results obtained with the E. coli cells infected with the wild type M13 phage particles in order to see if the mutation had any effect on the constract's ability to produce phage particles. However, plaque formation was observed in both experiments and the number of plaques observed in each group were comparable. This result showed us that the mutation did not affect the function of M13 genes present in plasmid pBlu5'recA M13 (figure 3.11).

Generation of an extra EcoRI site in the pBlu5'recA M13 plasmid prevented the transfer of the 5'recA-M13ΔIG fragment on this plasmid to pUC18 vector. Thus, the initial strategy for the construction of the recombination cassette had to be modified. In the new strategy, pBlue5'recA M13 and PCRII 3'recA Tet plasmids were digested with *Apa*I and *Sac*I restriction enzymes and 5'recA-M13ΔIG, and 3'recA-Tet fragments were purified after the fragments were seperated by the agarose gel electrophoresis. These two fragments were then ligated to each other to form a circular double stranded DNA molecule called "5'recA M13-3'RecA Tet" and used as the recombination cassette (figure 3.11). Since this molecule does not have a replication origin, it was not expected to be maintained in the cell as an independent plasmid upon its transfer into E. coli. Any tetracycline resistant colonies formed after the transformation of E. coli JM110 (recA+, F+) strain by electroporation would result from the integration of the molecule 5'recA M13-3'RecA Tet into the bacterial chromosome by homologous recombination (see figure 3.1a).

A-)



B-)
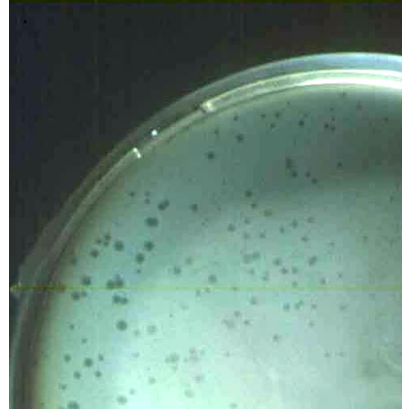


C-)

**Figure 3.11:** Results of plaque formation assay A-) Pblu5'recA-M13 B-) M13 mp19 as positive control C-) Pbluescript KS+as negative control
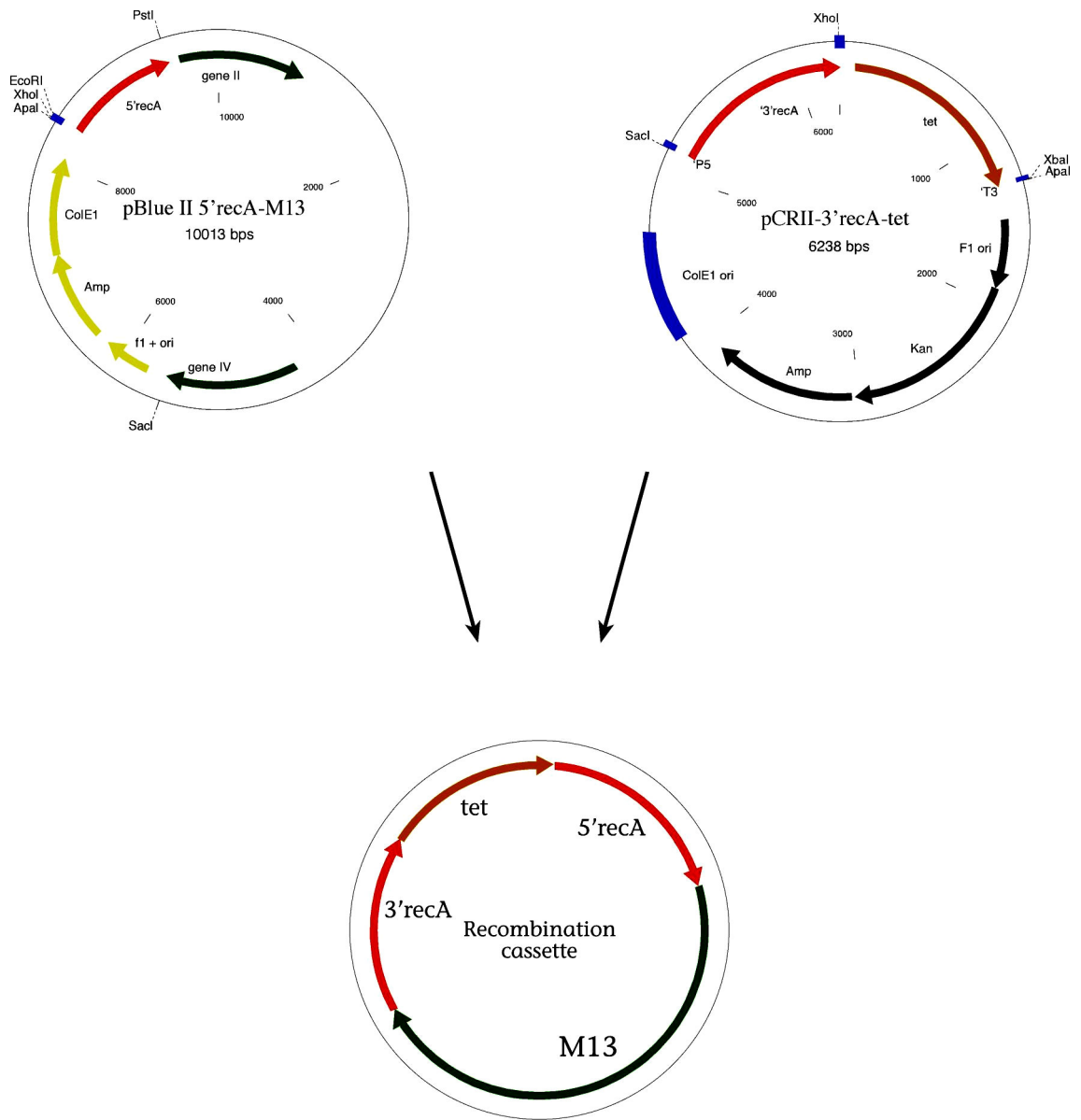
**Figure 3.12**: Schematic representation of double stranded DNA molecules

### 3.3.1 Homologous Recombination

Upon transformation of E. coli JM110 cells with the circular double stranded DNA molecule "5'recA M13 3'recA Tet" three tetracycline resistant colonies were observed after 48 hour of incubation. As described above, these colonies are expected to contain the 5'recA M13 3'recA Tet sequences integrated to their chromosomes. The possibility of the recombination events responsible for the integration are illustrated in figure 3.12. To prove the presence of the M13 genes in the *E.coli* chromosome, those three colonies were further analyzed by observing their plasmid profiles and PCR.

In order to show that the tetracycline resistance did not orginate from a self replicating plasmid molecule inside the cells, plasmid DNA isolation was performed to all the three tetracycline resistant cells. The results of this isolation experiment did not show any evidence for the presence of a plasmid molecule in any of the colonies (data not shown).
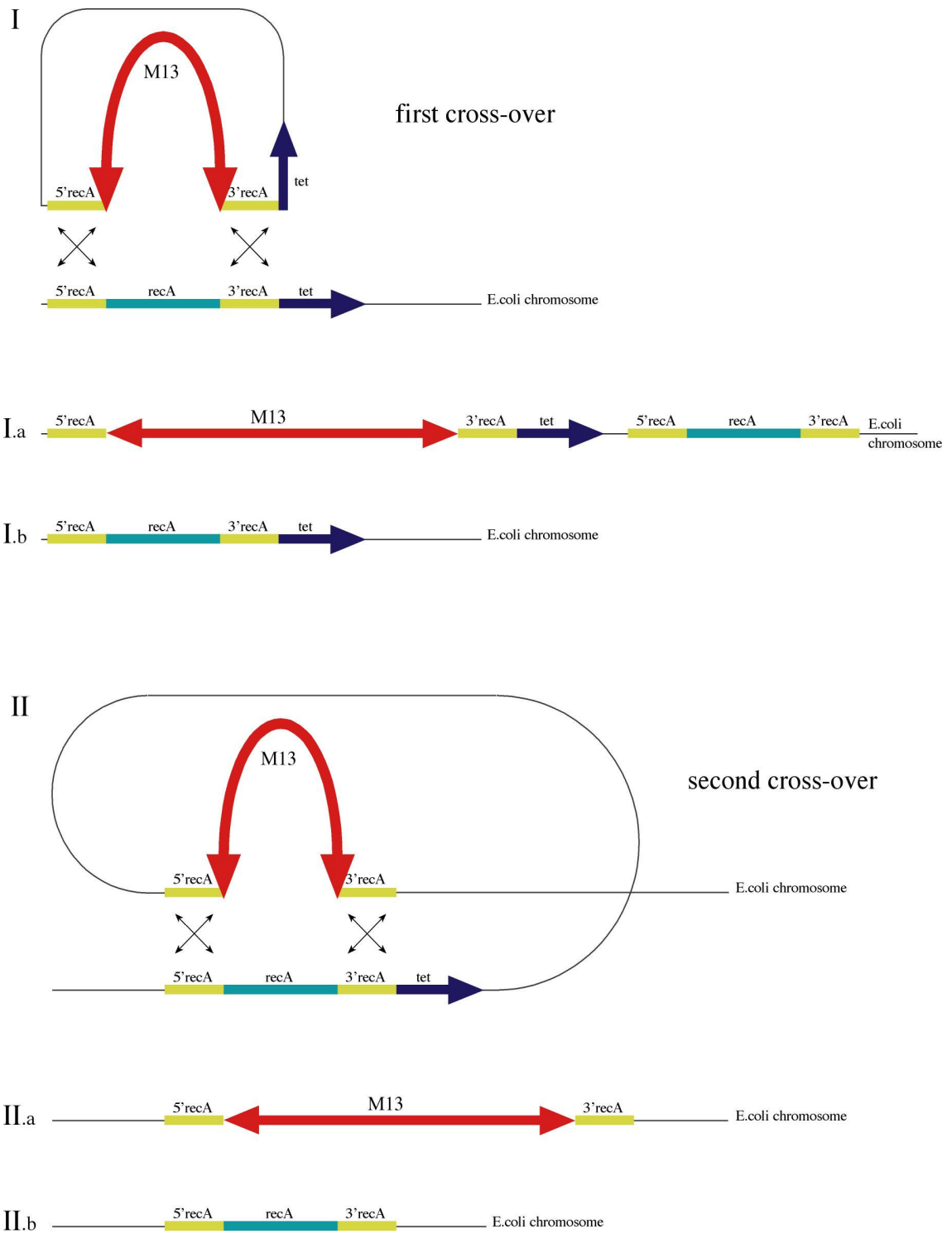
**Figure 3.13**: Schematic representation of created homologous recombination events
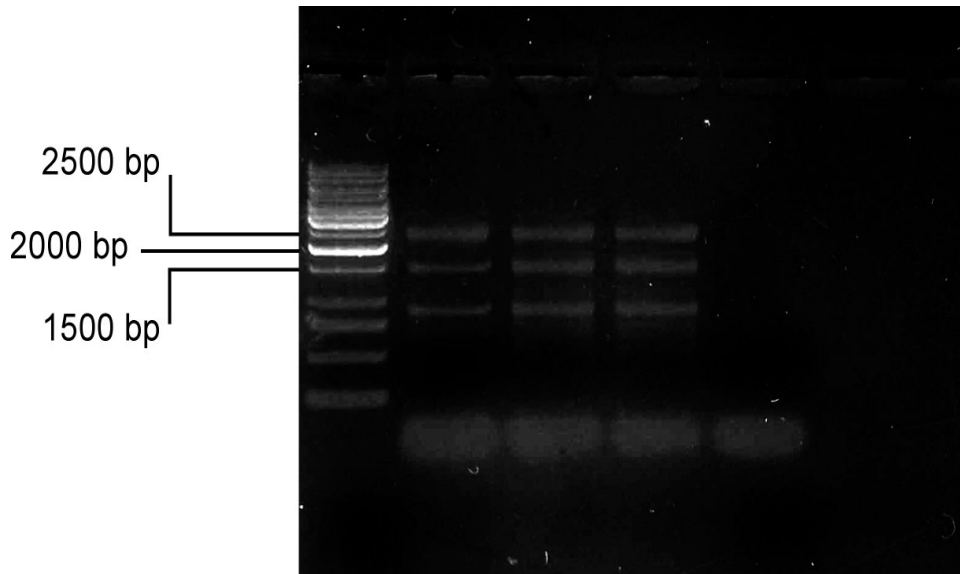
**Figure 3.14:** PCR analysis of recombinant chromosomal DNA for the presence of 5'recA-Tet fragment. Lanes 1. 1 kb DNA marker; 2. Recombinant colony 1; 3 recombinant colony 2; 4 Recombinant colony 3; 4. JM109 as negative control by using the primers T5, P35
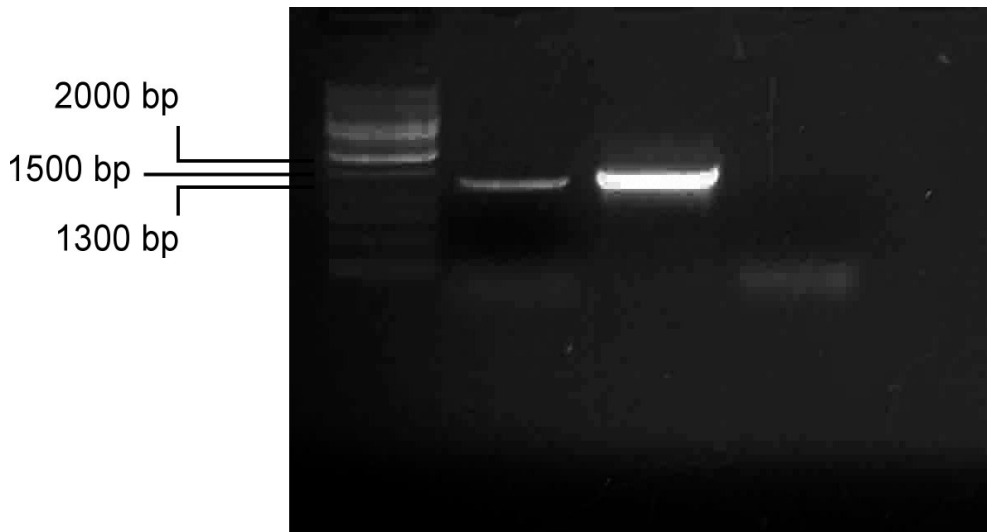


**Figure 3.15:** PCR analysis of recombinant chromosomal DNA for the presence of Tet marker gene. Lanes 1. 1 kb DNA marker; 2. Recombinant colony 1 3; PCRII 3'recA Tet as positive control; 4. JM109 as negative control by using the primers T3 and T5.
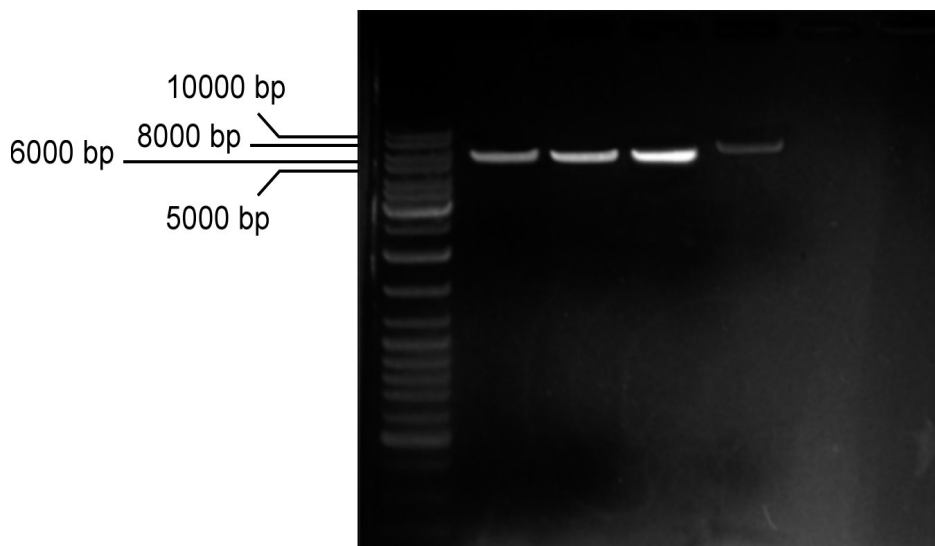
**Figure 3.16:** PCR analysis of recombinant chromosomal DNA for the presence of M13ΔIG. Lanes 1. 1 kb DNA marker; 2. Recombinant colony 1; 3 recombinant colony 2; 4. M13mp19 as positive control; 5. Pblu 5'recA M13 as positive control; 6. JM109 as negative control by using the primers M3,M5

Further analysis on these cells were performed by PCR by using primers that would amplify the sequences of the tetracycline resistance gene, M13ΔIG, and the combined sequences of tetracycline and 5'recA which would only be present in the integrated recombination cassette (figure 3.11). Upon PCR, the existence of bands corresponding to the fragments tet-5'rec A in all three recombinants showed the presence of the recombination cassette (See Figure 3.13). However, the M13ΔIG fragment was amplified in only the two of the recombinants (figure 3.14) and the tet sequence was amplified in only one of the recombinants (figure 3.15). This inconsistency could be partly explained by the impurities present in the genomic DNA templates used for PCR, affecting the efficiencies of the different primer pairs or by experimental errors.

In the observation of PCR product of M13ΔIG amplification reaction, a different profile was observed on positive control well. Two positive controls were used in this reaction as mentioned above. This time however, in the positive control M13ΔIG amplifications an unexpected product profile was obtained; it produced a much larger DNA fragment than the integrated fragment

The confirmation of the functionality of the M13 genes was tested for the formation of plaques on recombinant colonies transformed with pBluscriptKS+

52

phagemid DNA. If the cells expressed all the M13 genes in a functional form, those cells would be expected to form phage particles packaging the pBluscriptKS+ phagemid DNA and would give rise to plaque formations in a plaque assay.

However, although plaques were observed in the control assays where E. coli JM110 cells were infected with the wild type M13 phages, no plaque formation was observed in any of the recombinants during these assay.

# CHAPTER 4

# DISCUSSION

In this study, a new platform was aimed to be developed for the phage display technology. The strategy was novel and its advantages could have been enormous. However, although M13 genome that contained a deletion of the intergenic region was integrated into JM110 *E.col*i chromosome which was proved by number of analysis, M13 genome in *E.coli* chromosome did not support the production of phage particles upon transformation of the E. coli cells with a phagemid vector.

There have been many reports described so far related to the instability of M13 genome and the illegitimate recombination events that occured during the cloning experiments of M13 (Villette *et al*., 1996; French *et al*., 1992; Liu and Alberts, 1995). Phage display technology itself also suffers from deletion and insertion of random sequences and the loss of the genes coding for the desired polypeptides displayed on the phage surfaces Also, there are several reports describing the toxicity of some of the M13 proteins to the E. coli cells (Russel *et al*.,2004; Sidhu, 2000).

Thus it is highly possible that the insertion of a large DNA fragment containing the M13 genome together with the tetracycline resistance gene could have put the cells under a considerable strain interfering with their viability and forcing the integrated M13 sequences to mutate. This would ultimately lead to the selection of integrants carrying mutations leading to the loss functional M13 genes preventing the formation of phage particles.

We also observed some mutations during the cloning of M13 DNA during the preparation of the recombination cassette before the integration experiments. We cloned the M13 genome into three different plasmids which were pUC18, pCRXL-TOPO and pBluescriptKS+. Restriction analysis of the recombinant colonies obtained after the transformation of E. coli cells with the ligation mixtures of M13 genome with the relevant plasmids showed several insertions or deletions in the cloned plasmids (data not shown). However, we chose to continue our expirements by cloning in the pBluescriptKS+ plasmid (phagemid) because it seemed to form the most number of stable recombinant plasmids. Since pBluescript phagemid contains the intergenic region of M13, such recombinants can produce and release phage particles (see chapter 3,

figure 3.11) preventing the accumulation of phage proteins inside the cells that might be toxic for the cells. However, recombinants of the other plasmids cannot produce phage particles thus leading to the accumulation of toxic M13 proteins ultimately leading to cell death making the selection of only the cells carrying the mutated recombinant plasmids.

On the other hand, during the integration experiments, we had to use a construct that does not contain the intergenic region of the M13 genome in order to force the construct to integrate into the chromosome. However, since a bacteria that contains an integrated copy of this construct lacks an M13 intergenic region, it will fail to produce phage particles leading to the accumulation of M13 toxic proteins inside the cells causing cell death. Thus the only bacteria that will be selected would be the ones which do not express the toxic M13 proteins due to mutations gained after the integration event.

In order to overcome such problems the toxicities of each of the M13 genes on the E. coli cells should be investigated by cloning and expressing each of them separately in the cells. Then, the ones which are toxic should be eliminated from the recombination constracts and expressed in the phagemid genome that also carries the P3 and the P3-fusion genes. Only then we believe that this strategy we tried to be developed in this study will be a success and a novel alternative to the current phage display strategies.

# REFERENCES

Aguilera, A., 2002. " The connection between transcription and genomic instability", *European Molecular Biology Organization*, Vol. 21, pp. 195- 201.

Al- bukhari, T.A.M.A., Tighe, P. and Todd, I., 2002." An immuno-precipitation assay for determining specific interactions between antibodies and phage selected from random peptide expression libraries", *Journal of Immunological Methods*, Vol. 264, pp. 163- 171.

Asano, S., Higashitani, A. and Horiuchi, K. 1999." Filamentous phage replication initiator protein gpII forms a covalent complex with the 5' end of the nick it introduced", *Nucleic Acids Research*, Vol. 27, pp. 1882-1889.

Ausubel, F.M, Bent, R., Kingston, R.E, Moore, D.D., Seidman, J.G. Smith, J.A.,Struhl, K. 1997 "Current Protocols in Molecular Biology*" Wiley, New York, USA.*

Azzazy, H.M.E. and Highsmith, W.E., 2002. "Phage display technology: applications and recent innovations", *Clinical Biochemistry*, Vol. 35, pp. 425-445.

Baek, H., Suk, K., Kim, Y. and Cha, S., 2002. "An improved helper phage system for efficient isolation of specific antibody molecules in phage display", *Nucleic Acids Research*, Vol. 30, pp. 1-9.

Bai, Y.and Feng, H., 2004." Selection of stably folded proteins by phage-display with proteolysis", *Eur.J. Biochem.*, Vol. 271, pp.1609-1614.

Barlett, M. M., Erickson, M. J. and Meyer, R. J., 1990. " Recombination between directly repeated origins of conjugative transfer cloned in M13 bacteriophage DNA models ligation of the transferred plasmid strand", *Nucleic Acids Research,* Vol. 18, pp. 3579- 3586

Bishop, A. J. R. and Schiestl, R. H., 2000. " Homologous recombination as a mechanism for genome rearrangements: environmental and genetic effects", *Human Molecular Genetics,* Vol. 9, pp. 2427-2434.

Blum, P., Holzschu, D., Kwan, H., Riggs, D. and Artz, S., 1989. "Gene replacement and retrieval with recombinant M13mp bacteriophages", *Journal of bacteriology*, Vol. 171, pp. 538-546.

Boyd, D., Weiss, D. S., Chen, J. C. and Beckwith, J., 2000. " Towards single-copy gene expression systems making gene cloning physiologically relevant: lambda InCh, a simple *Escherichia coli* plasmid-chromosome shuttle system", *Journal of Bacteriology*, Vol. 182, pp. 842-847.

Chappel, J.A., He, M., Kang, A.S., 1998. Modulation of antibody display on M13 filamentous phage. J. *Immunological Methods,* Vol. 221, pp. 25-34.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G., 1991." Making antibody fragments using phage display libraries", *Nature*, Vol. 352, pp.624-628.

Cleary, J. M. and Ray, D. S., 1980." Replication of the plasmid pBR322 under the control of cloned replication origin from the single-stranded DNA phage M13", *Proc. Natl. Acad. Sci.*, Vol. 77, pp. 4638-4642.

Court, D. L., Sawitzke, J. A. and Thomason, L. C., 2002 " Genetic engineering using homologous recombination", *Annual Review of Genetics*, Vol. 36, pp. 361-388.

Danner, S. and Belasko, J. G.,2001."T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries", *Proc. Nat. Acad. Sci.*, Vol. 23, pp. 12954-12959.

D'Mello, F. and Howard, C.R., 2001. " An improved selection procedure for the screening of phage display peptide libraries", *Journal of Immunological Methods,* Vol.247, pp. 191-203.

Dotto, G. P., Horiuchi, K., Jakes, K. S. and Zinder, N. D., 1982." Replication origin of bacteriophage f1: two signals required for its function", *Journal of Molecular Biology*, Vol. 162, pp. 335- 343.

El Karoui, M., Amudsen, S. K., Dabert, P. and Gruss, A., 1999. "Gene replacement with linear DNA in electroporated wild-type *Escherichia coli", Nucleic Acids Research*, Vol. 27, pp. 1296- 1299.

Felici, F., Castagnoli, L., Musacchio, A., Japelli, R.,Cesareni, G., 1991. "Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector". *Journal of Molecular Biology*. Vol. 222, pp. 301-310

Fernandez-Gacio, A., Uguen, M. and Fastrez, J.2003. " Phage display as a tool for the directed evolution of enzymes", *Trends in Biotechnology*, Vol. 21, pp. 408-414.

Fernandez-Gacio, A., Uguen, M. and Fastrez, J.2003. " Phage display as a tool for the directed evolution of enzymes", *Trends in Biotechnology*, Vol. 21, pp. 408-414.

Fuh, G. and Sidhu, S. S., 2000. " Efficient phage display of polypeptides fused to the carboxy-terminus of the M13 gene-3 minor coat protein", *FEBS Letters*, Vol. 480, pp. 231-234.

Fujioka, K., Aratani, Y., Kusano, K. and Koyama, H., 1993." Targeted recombination with single-stranded DNA vectors in mammalian cells", *Nucleic Acids Research,* Vol.21, pp. 407-412.

Gao, C., Mao, S., Lo, C. L., Wirsching, P., Lerner, R. A. and Janda, K. D., 1999." Making artificial antibodies: a format for phage display of combinatorial heterodimeric arrays", *Proc. Natl. Acad. Sci.*, Vol. 96, pp. 6025- 6030.

Gaskin, D. J. H., Starck, K., Turner, N. A. and Vulfson, E. N., 2001. " Phage display combinatorial libraries of short peptides: ligand selection for protein purification", *Enzyme and Microbial Technology*, Vol. 28, pp. 766-772.

Greenwood, J., Willis, A. E. and Perham, R.N., 1991." Multiple display of foreign peptides on a filamentous bacteriophage", *Journal of Molecular Biology*, Vol. 220, pp. 821-827.

Griffiths, A. D., Malmqvist, M., Marks, J. D., Bye, J. M., Embleton, M. J., McCafferty, J., Gorick, B.D., Hughes-Jones, N.C., Hoogenboom, H. R. and Winter, G. P., 1993." Human anti self-antibodies with high specificity from phage display libraries, *European Molecular Biology Organization*, Vol. 12, pp. 725-734.

Hamilton, C. M., Aldea, M., Wasburn, B. K., Babitzke, P. and Kushner, S. R., 1989. " New methods for generating deletions and gene replacements in *Escherichia coli"*, *Journal of Bacteriology,* Vol. 171, pp. 4617-4622.

Held, H. A. and Sidhu, S. S., 2004. "Comprehensive mutational analysis of the M13 Major coat protein: Improved scaffolds for C- terminal phage display", *Journal of Molecular Biology*, pp. 1-11.

Hoess, R. H., 2001. "Protein design and phage display" *Chemical Reviews*, Vol. 101, pp.3205-3218.

Holliday, R., 1964." A mechanism for gene conversion in fungi", *Genet. Res.*, Vol. 5, pp. 282- 304.

Hoogenboom, H.R. and Winter, G., 1992." By-passing immunization. Human antibodies from synthetic repertories of germline VH segments rearranged *in vitro"*, *Journal of Molecular Biology*, Vol. 227, pp. 381-388.

Hoogenboom, H.R., 1997. Designing and optimizing library selection strategies for generating high-affinity antibodies. TIBTECH15, 62-70.

Hoogenboom, H.R., De Bruine, A. P., Hufton, S. E., Hoet, R.M., Arends,J.W. and Rovers, R.C., 1998." Antibody phage display and its applications", *Immunotechnology*, Vol.4, pp.1-20.

Hoogenboom, H.R., and Charmes, P., 2000." Natural and designer binding sites made by phage display technology", *Immunol. Today*, Vo. 21, pp. 371-378.

Hufton, S.E., Moerkerk, P., Muelemans, E.V., de Bruine, A., Arends, A., Hoogenboom, H.R., 1999. "Phage display of cDNA repertoires: the pVI display systemand its applications for the selection of immunogenic ligands*", J. Immunol. Methods,* Vol. 231, p. 39.

Jestin, J., Volioti, G. and Winter, G., 2001." Improving the display of proteins on filamentous phage", *Res.Microbiol.,* Vol. 152, pp. 187-191.

Jung, S., Honegger, A. and Plückthun, A., 1999. "Selection for improved protein stability by phage display", *Journal of Molecular Biology*, Vol. 294, pp. 163- 180.

Johns, M., George, A.J.T. and Ritter, M.A., 2000. *"In vivo* selection of scFv from phage display libraries", *J. Immunol. Meth.*, Vol. 239, pp. 137-151.

Kay, K.B., Kasanov, J., Yamabhai, M., 2001. "Screening phage-display combinatorial peptide libraries". Methods Vol. 24, pp. 240-246.

Kim, M. H. and Ray, D. S., 1985. " Mutational mechanisms by which an inactive replication origin of bacteriophage M13 is turned on are similar to mechanisms of activation of *ras* proto-oncogenes", *Journal of Virology*, Vol.53, pp. 871-878.

Kramer, R.J., Cox, F., Horst, M.V., Oudenjin, S.V., Res, P.C.M., Bia, J., Logtenberg, T. and Kruif, J, 2003. "A novel helper phage that improves phage display selection efficiency by preventing the amplification of phages without recombination protein", *Nucleic Acids Research*, Vol. 31, pp. 1-9.

Kowalczykowski, S. C., 1991." Biochemistry of genetic recombination: energetics and mechanism of DNA strand exchange", *Annu.Rev.Biophys. Chem.*, Vol.20, pp.539-575.

Larocca, D., Jensen-Pergakes, K., Burg, M.A., Baird, A., 2001. "Receptor-targeted gene delivery using multivalent phagemid particles", *Mol. Ter.*, Vol. 3, p. 476

Li, M., 2000." Applications of display technology in protein analysis", *Nature Biotechnology*, Vol. 18, pp. 1251-1256.

Makowski, L., 1992. "Terminating a macromolecular helix structural model for minor proteins of bacteriophage M13", Journal of Molecular Biology, Vol. 228, pp.885-892.

Marvin, D. A., 1998. " Filamentous phage structure, infection and assembly", *Current Opinion in Structural Biology,* Vol. 8, pp. 150-158.

McCafferty, J., Griffiths, A. D., Winter, G., Chiswell, D. J., 1990. "Phage antibodies filamentous phage displaying antibody variable domains", Nature, Vol. 348, pp.552-554.

McCafferty, J., 1996." Phage Display: factors affecting panning efficiency", *A laboratory Manual. Academic Press Inc., San Diego, CA*, pp. 261-276.

McCafferty, J. and Johnson, K.S., 1996." Construction and screening of antibody display libraries. In: B.K. Kay, J. Winter and J. McCafferty (eds), Phage display of peptides and proteins", *Academic press*, pp. 79- 111.

Meselson, M. S. and Radding, C. M., 1975." A general model for genetic recombination", *Proc. Natl. Acad. Sci.,* Vol.72, pp. 358-361.

Messing, J., Gronenborn, B., Müller-Hill, B. and Hofschneider, P. H., 1977." Filamentous coliphage M13 as a cloning vehicle: insertion of a *HindII* fragment of the *lac* regulatory region in M13 replicative form *in vitro"*. *Proc. Natl. Acad. Sci,* Vol. 74, pp. 3642-3646.

Nilsson, N., Malmborg, A. C. and Borrebaeck, C. A. K., 2000. "The phage infection process: a functional role for the distal linker region of bacteriophage protein 3", *Journal of Virology*, Vol. 74, pp. 4229- 4235.

Nissim, A., Hoogenboom, H.R., Tomlinson, I. M., 1994. "Antibody fragment from a single pot phage display library as immunochemical reagents", *EMBO J,* Vol. 13, pp. 692-698.

Parmerly, S., F. and Smith, G. P., 1988." Antibody-selectable filamentous fd phage vectors: affinity purification of target genes", *Gene*, Vol. 73, p. 305.

Petrenko, V. A. and Vodyanoy, V. J., 2003." Phage display for detection of biological treat agents", *Journal of Microbiological Methods*, Vol. 53, pp. 253- 262.

Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P. and Neri, D., 1998. "Design and use of a phage display library", *Journal of Biological Chemistry*, Vol. 273, pp. 21769-21776.

Pini, A. and Bracci, L., 2000." Phage display of Antibody fragments", *Current Protein and Peptide Science*, Vol.1, pp. 155-169.

Rakonjac, J., Jovanovich, G. and Model, P., 1993." Filamentous phage infection-mediated gene expression: construction and propagation of the geneIII deletion mutant helper phage*", Gene*, Vol.198, pp. 99-103.

Rhyner, C., Kodzius, R. and Crameri, R., 2002. " Direct selection of cDNAs from filamentous phage surface display libraries: potential and limitations", *Current Pharmaceutical Biotechnology,* Vol. 3, pp. 13-21.

Rodi, D. J. and Makowski, L., 1999." Phage display technology-finding a needle in vast molecular haystack", ", *Current Opinion Biotechnology,* Vol. 10, pp. 87- 93.

Rodi, D. J., Makowski, L. and Kay, B. K., 2001." The benefits of phage display in molecular-recognition studies", *Current Opinion Chemical Biology*, Vol.6, pp. 92-96.

Rondot, S., Koch, J., Breitling, F.and Dübel, S., 2001." A helper phage to improve single-chain antibody presentation in phage display", *Nature Biotechnology*, Vol. 19, pp. 75-78.

Rosander, A., Bjerketorp, J., Frykberg, L. and Jacobsson, K., 2002." Phage display as a novel screening method to identify extracellular proteins", *Journal of Microbiological Methods*, Vol. 51, pp. 43- 55.

Russel, M., 1991."Filamentous phage assembly", *Molecular Microbiology*, Vol. 5, pp. 1607-1613.

Russel, M., 2004." Introduction to phage display In: H. Lowman, T. Clackson, (eds), Phage Display: A Practical Approach", *Oxford University Press,* pp. 1-67

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989." Molecular cloning: a laboratory manual", *Cold Spring Harbor Laboratory press, NY.*

Sblattero, D., Lou, J., Marzari, R. and Bradbury, A., 2001. "In vivo recombination as a tool to generate molecular diversity in phage antibody libraries", *Reviews in Molecular Biotechnology*, Vol. 74, pp. 303- 315.

Scott, J. K. and Smith, G. P., 1990. "Searching for peptide ligands with an epitope library", *Science*, Vol. 249, p. 386.

Scott, J. K., 2001. "Filamentous Bacteriophages In: D.R. Burton, J.K. Scott, G. Silverman, C.F. Barbas (eds), Phage Display: A Laboratory Manual", *Cold Spring Harbor Laboratory Press,* pp. 227-253.

Shibata, T., 2001. " Functions of homologous DNA recombination", *Focused on Bioarchitect Research,* No. 41, pp.21-23.

Sidhu, S. S., Weiss, G. A. and Wells, J. A., 2000. " High copy display of large proteins on phage for functional selections", *Journal of Molecular Biology*, Vol. 296, pp. 487-495.

Sidhu, S. S., 2001." Engineering M13 for phage display", *Biomolecular Engineering*, Vol. 18, pp. 57- 63.

Smith, G.P., 1985. "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surfaces". *Science,* Vol. 228, pp. 1315-1317.

Smith, G.P., Scott, J.K., 1993." Libraries of peptides and proteins displayed on filamentous phage", *Methods Enzymology*, Vol. 217, pp. 228-257

Smith, G.P. and Petrenko, V. A., 1997." Phage display", *Chemical Reviews*, Vol. 97, pp. 391-410.

Soltes, G., Barker, H., Marmai. K., Pun, E., Yuen, A. and Wiersma, E.J., 2003. "A new helper phage and phagemid vector system improves viral display of antibody Fab fragments and avoids propagation of insert-less virions", *Journal of Immunological Methods*, Vol. 274, pp. 233-244.

Sparks, A. B., Adey, N. B., Cwirla S.and Kay, B. K., 1996." Screening phage-display random peptide libraries. In: B.K. Kay, J. Winter and J. McCafferty (eds), Phage display of peptides and proteins", *Academic press*, pp. 227-253

Stoop, A.A., Jespers, L., Lasters, I., Eldering, E., Pannekoek, H., 2000 " High density mutagenesis by combined DNA shuffling and phage display to assign essential amino acid residues in protein-protein interactions: application to study structure-function of plasminogen activator inhibitor" *Journal of Molecular Biology*, Vol.301, p. 1135

Stopar, D., Spruijt, R. B., Wolfs, C. J. A. M. and Hemminga, M. A., 2003." Protein-lipid interactions of bacteriophage M13 major coat protein", *Biochimica et Biophysica Acta,* Vol. 1611, pp. 5-15.

Weiss, G. A., Roth, T. A., Baldi, P. F. and Sidhu, S. S., 2003." Comprehensive mutagenesis of the C-terminal domain of the M13 gene-3 minor coat protein:the requirements for assembly into the bacteriophage particle", *Journal of Molecular Biology*, Vol.332, pp. 777- 782.

Willats, W. G. T., 2002." Phage display: practicalities and prospects", *Plant Molecular Biology,* Vol. 50, pp. 837- 854.

Williats, W. G.T., Steele-King, C.G. and Knox, J.P., 2002 "Antibody techniques", *Oxford University Press, Oxford, U. K.*

Winter, G., Griffiths, A. D., Hawkins, R. E. and Hoogenboom, H. R., 1994. "Making antibodies by phage display technology", *Annual Reviews of Immunology*, Vol.12, pp. 433-455.

Winter, G.1998."Synthetic human antibodies and a strategy for protein engineering", *FEBS letters*, Vol.430, pp. 92-94.

Winter, G.1998."Antibody and peptide ligands by repertoire selection technologies", *Journal of Molecular Recognition*, Vol.11, pp. 126-127.

Vaughan, T.G., Osbourn, J.K., Tempest, P.R., 1998," Human antibodies by design", *Nature Biotechnology,* Vol. 16, p. 309.

Zhang, W. and Evans, D. H., 1993. "DNA strand exchange catalyzed by proteins from vaccinia virus-infected cells", *Journal of Virology*, Vol. 67, pp. 204- 212.

# APPENDIX A

# CHEMICALS

**Table A.1 Chemicals Used in the Experiments (cont. on next page)**

| CHEMICAL | CODE |
|---|---|
| Agar-Agar | Applichem A0949 |
| Peptone from Casein | Applichem A2210 |
| D (+)-Glucose | Applichem A3666 |
| Yeast Extract | Merck 1.03753 |
| Glycerol | Applichem A2926 |
| Sodium chloride | Applichem A2942 |
| Potassium phosphate | Applichem A2945 |
| $MgSO_4.7H_2O$ | Merck 1.05886 |
| Disodium hydrogen phosphate | Applichem A2943 |
| Ammonium sulfate | Applichem A3585 |
| Cetyl trimethylammonium bromide | Applichem A0805 |
| Tris Base | Sigma T6066 |
| EDTA | Applichem A2937 |
| Isopropanol | Applichem A3928 |
| Proteinase K | Applichem A3830 |
| Ethidium bromide | Applichem A1151 |
| Ethanol | Applichem A1151 |
| Taq DNA polymerase | MBI, Fermentas EP0401 |
| EcoRI | MBI, Fermentas ER0273 |
| SacI | MBI, Fermentas ER1133 |
| PstI | MBI, Fermentas ER0612 |
| ApaI | MBI, Fermentas ER1412 |
| XhoI | MBI, Fermentas ER0692 |
| XbaI | MBI, Fermentas ER0682 |
| T4 DNA Ligase | MBI, Fermentas EP0062 |
| CIAP | MBI, Fermentas EF0342 |

| | |
|---|---|
| dNTP set | MBI, Fermentas R0181 |
| Ampicillin | Applichem A0839 |
| Kanamycin | Applichem A1493 |
| Tetracycline | Applichem A2228 |
| X-Gal | Applichem A4978 |
| IPTG | Applichem A4773 |
| Agarose (Standard) | Applichem A2114 |
| Lysozyme | Applichem A3711 |
| Chloroform | Applichem A3830 |
| Isoamyl alcohol | Applichem A2610 |
| Sodium dodecyl sulphate | Applichem A2263 |
| Sodium hydroxide | Merck 1.06498 |
| Hydrochloric acid (HCl) | Merck 1.00317 |
| Ammonium acetate | Applichem A2936 |
| Phenol | Applichem A1594 |
| 1 kb DNA Ladder Gene Ruler$^{TM}$ | Fermentas SM0313 |
| D (+)-Sucrose | Applichem A3935 |
| Potassium hydroxide | Amresco 1073B60 |

# APPENDIX B

# RECIPIES FOR MEDIA USED IN EXPERIMENTS

## B.1. LURIA- BERTANI BROTH AND AGAR (LB)

g/l

Deionized $H_2O$, to 950 ml

Tryptone, 10

Yeast extract, 5

NaCl, 10

For solid medium

Bacto-agar, 13

To prepare LB medium, ingredients are dissolved by stirring until the solutes have dissolved. The pH is adjusted to 7.0 with 1M NaOH. The volume of the solution is completed to 1 liter with deionized $H_2O$. Medium is sterilized by autoclaving for 20 m at 15 psi on liquid cycle.

## B.2. SOC MEDIUM

g/l

Deionized $H_2O$, to 950 ml

Tryptone, 20

Yeast extract, 5

NaCl, 0.5

For solid medium

Bacto-agar, 13 g

Components are added into distilled water and mixed thoroughly. The pH is brought to 7.3. Medium is sterilized by autoclaving 20 m at 15 psi on liquid cycle. After sterilization 20 ml of a sterile 1M solution of glucose (This solution is made by 18 g of

glucose in 90 ml of $H_2O$ and after the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized $H_2O$ and sterilized by filter)

## B.3. MINIMAL MEDIUM (M9)

## B.3.A. 5X M9 MEDIUM

g/l

$Na_2HPO_4$, 30
$KH_2PO_4$, 15
$NH_4Cl$, 5
$CaCl_2$, 0.15 (optional)

## B.3. M9 PLATE

15 g of agar is autoclaved in 800 ml of deionized $H_2O$ for 15 m. sterile concentrated minimal medium and carbon source was added into the sterile agar solution.

## B.4. 2X YT MEDIUM

Deionized $H_2O$, to 950 ml
Tryptone, 16
Yeast extract, 10
NaCl, 5
For solid medium
Bacto-agar, 13
For soft agar
Bacto-agar, 8

# APPENDIX C

# BUFFERS AND STOCK SOLUTIONS

.

## C.1. 50 X TAE

242 g Tris base is dissolved in deionized water; 57.1 ml glacial acetic acid and 100ml 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionized water.

## C.2. 1 X TAE

20 ml of 50X TAE buffer is taken and the volume is adjusted to 1000 ml with deionized water to obtain 1000 ml 1X TAE buffer.

## C.3. 10 X TBE

108 g Tris Base and 55 g boric acid were weighed. They were dissolved in nearly 800 ml of deionized water and 40 ml 0,5 M EDTA pH 8,0 was added. The volume was brought to 1 L with deionized water

## C.4. 1X TBE

100 ml 10X TBE was taken and the volume was brought to 1 liter with deionized water to obtain 1liter 1X TBE buffer

## C.5. CTAB/NaCl (10% CTAB in 0.7M NaCl)

NaCl (4.1 g) is dissolved in 80 ml water and CTAB is added slowly while heating and stirring. Final volume is adjusted to 100 ml

## C.6.  1X TE (pH 8.0)

10 mM Tris (pH 8.0), 1mM EDTA

## C.7. Tris-HCl (1 M, pH 8.0)

121.1 g Tris base is dissolved in 800 ml of deionized water. pH is adjusted to 8.0 with concentrated HCl. Volume is adjusted to 1000 ml with deionized water. The solution is sterilized by autoclaving.

## C.8. Tris-HCl (1M, pH 7.2)

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 7.5 with concentrated HCl. Volume is brought to 1l with deionized water.

## C.9. EDTA (0.5 M, pH 7.5, 8.0 and 9.5)

186.1 g of EDTA is dissolved in 800 ml of deionized water and pH is adjusted to desired value with 10 N NaOH. Volume is brought to 1000 ml with deionized water. The solution is sterilized by autoclaving.

## C.10. Sodium Acetate (3M, pH 4.8)

408.1 g sodium acetate (3 H2O) is dissolved in 800 ml deionized water and the pH is adjusted to 4.8 by glacial acetic acid. Volume is adjusted to 1000 ml with distilled water. The solution is sterilized by autoclaving.

## C.11. Solution I

50 mM glucose
10 mM EDTA (pH 8.0)
25 mM Tris.Cl (pH 8.0)
Solution I is prepared in 100 ml of deionized $H_2O$ and sterilized by autoclaving for 20 m at 15 psi on liquid cycle.

## C.12. Solution II

0.2 N NaOH (freshly diluted from 10 N stock)
1% SDS

## C.13. Solution III

5M Potassium Acetate
Glacial acetic acid
$H_2O$
The resulting solution is 3M with respect to potassium and 5M with respect to acetate

## C.14. Ammonium Acetate (10M)

770 g of ammonium acetate is dissolved in 800 ml of distilled water. Volume is adjusted to 1000ml. The solution is sterilized by filtration.

## C.15. Ethidium Bromide (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionized water by stirring for several hours. The solution is stored in a dark bottle at room temperature.

## C.16. Phenol

Phenol should be allowed to warm at room temperature, and it is melted at 68 °C. Equal volumes of buffer (usually 0.5 M Tris.Cl, pH 8.0, at room temperature) are added to the melted phenol. The mixture is stirred for 15 minutes and allowed to settle. When the two phases have separated, the aqueous (upper) phase is removed using a separation funnel. Then equal volume of 0.1 M Tris.Cl, pH 8.0, is added to the phenol. The mixture is again stirred for 15 minutes and allowed to settle. The aqueous phase is removed as described before. The extractions are repeated until the pH of the phenolic phase reached to7.8. The pH is measured by using pH paper slips. After the phenol is equilibrated, the mixture is divided into aliquots. They are stored less than 100 mM

Tris.Cl (pH 8.0) at -20ºC. Before use, the phenol is melted at room temperature. Hydroxyquinoline and β-mercaptoethanol are added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at 4ºC.

## C.17. Chloroform-Isoamyl Alcohol Solution

96 ml of chloroform was mixed with 4 ml of isoamyl alcohol.
.

## C.18. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Equal volume of phenol and chloroform isoamyl alcohol (24:1) solutions are mixed. The solution is stored in a light-tight bottle at +4°C for periods up to 1 month.

## C.19. 6X Gel Loading Buffer (20 ml)

2 ml of 10x TBE, 6 ml of glycerol and 12 ml deionized water are mixed. Bromophenol blue is added with toothpick until obtaining sufficient color of the solution.

## C.20. Polyethylene Glycol (PEG) Solution

30 % (w/v) PEG 8000
1.6 M NaCl
Store indefinitely at +4 $^{0}$C.

# APPENDIX D

# PCR RECIPIES

## D.1 PCR MIXTURE

Mg free Taq DNA polymerase buffer: 5 µl

$MgCl_2$ (25 mM) :3 µl

Sterile deionized water: 35 µl

Oligo forward (10 picomole/ µl): 1 µl

Oligo reverse (10 picomole/ µl): 1 µl

dNTP (2mM each) 10X: 5 µl

Taq DNA polymerase: 0.25 µl (1.25 U)

## D.2 6X GEL LOADING BUFFER (20 ML)

10x TBE 2 ml

Glycerol 6 ml

Deionized water 12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

## D.3 dNTP (10X)

10 µl of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0,2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at 20 °C.

## D.4. RESTRICTION ENZYME MIXTURE

Restriction enzyme buffer: 5 µl

Sterile deionized water: 35µl

DNA: 10 µl

Restriction Enzyme: 0,5 µl (5U)

# APPENDIX E

# OLIGONUCLEOTIDE PRIMERS


**P35** **:**CCT TCT CGG AGC TCA TCA AGT GTT TTG TAG AAA TTG TTG CC

**P33** **:**CCT TCT CGC TCG AGG CTC AAT CTG AAA GGT TCC TTT GCC

**P55** **:**CCT TCT CGG AAT TCC ATC GTG TTT GAA ATA GTC GCC G

**P53** **:**CCT TCT CGC TGC AGG CAA CCC GAA CTC AAC GCC GG

**M5** **:**CCT TCT CGC TGC AGT TTA ACG CGA ATT TTA ACA AAA TAT TAA CG

**M3** **:**CCT TCT CGG AGC TCC TAC AGG GCG CGT ACT ATG GTT GC

**T5** **:**CTT TCT CGC TCG AGT TCT CAT GTT TGA CAG CTT A

**T3** **:**CCT TCT CGT CTA GAT CAG GTC GAG GTG GCC C